

## Wound responses of wild apples suggest multiple resistance mechanism against blue mold decay



Wojciech J. Janisiewicz<sup>a,\*</sup>, Breyne Nichols<sup>a</sup>, Gary Bauchan<sup>c</sup>, Thomas C. Chao<sup>d</sup>, Wayne M. Jurick II<sup>b</sup>

<sup>a</sup> Appalachian Fruit Research Station, Agricultural Research Service, U.S. Department of Agriculture, 2217 Wiltshire Road, Kearneysville, WV 25430, United States

<sup>b</sup> Food Quality Laboratory, Henry A. Wallace Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, United States

<sup>c</sup> Electron and Confocal Microscopy Unit, Henry A. Wallace Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, United States

<sup>d</sup> Plant Genetic Resources Unit, Agricultural Research Service, U.S. Department of Agriculture, Geneva, NY 14456, United States

### ARTICLE INFO

#### Article history:

Received 21 September 2015

Received in revised form 1 December 2015

Accepted 4 December 2015

Available online 27 February 2016

#### Keywords:

Wild apple germplasm  
Resistance to *Penicillium*  
Postharvest decay

### ABSTRACT

Blue mold caused primarily by *Penicillium expansum* and to a lesser extent other *Penicillium* spp. is the most destructive disease of stored apples in the US and worldwide. It was recently shown that resistance to blue mold exists in wild apple germplasms, *Malus sieversii*, from Kazakhstan and in other species from different regions maintained as a collection in Geneva, NY. We initiated studies to determine the durability and the mechanism(s) of resistance to *P. expansum* in select wild apple accessions. Wound responses (up to 96 h in 24 h intervals), affecting *P. expansum* infection, and related cytological changes were determined in accessions with varying levels of resistance. In general, the more resistant the accession, the quicker the wound response that prevented the fungus from infecting tissue and causing decay. No decay developed on immune apple accessions, even when inoculated immediately after wounding at the inoculum concentration of  $10^5$  conidia/mL. On a moderately resistant accession, a 24 h interval between wounding and inoculation was sufficient to avert decay. Reactive oxygen species (ROS) were detected at high levels immediately after wounding in the immune as well as susceptible accessions. Callose and lignin/suberin appears to play a minor role in resistance responses. Our results indicate the presence of a high level of durable resistance/immunity in the wild apples which is governed by several mechanism(s). This presents a new challenge for explaining the observed resistance and at the same time creates an opportunity for exploiting these resistant mechanisms in breeding programs to incorporate resistance to fruit decays into commercial cultivars.

Published by Elsevier B.V.

### 1. Introduction

Apple is a major crop in the United States with an annual production of 6.3 billion pounds of fruit with a fresh market value of \$2.5 billion (USDA National Agricultural Statistics Service, 2014). Current production is based on a limited number of cultivars which face significant challenges with respect to control of diseases, pests, and adaptation to environmental stresses. There is a growing consumer demand for fruit free of pesticides (Volk et al., 2015). Significant progress has been made in finding alternatives to synthetic fungicides during the past three decades including

biological control, substances generally regarded as safe (GRAS), and various physical treatments (Janisiewicz and Korsten, 2001; Lurie et al., 1998; Ramanazzi et al., 2012; Soliva et al., 2014; Wilson et al., 1994). In general, these alternatives do not have as wide a spectrum of activity and curative ability as the synthetic fungicides; however, in combination they can provide consistent control under a broad spectrum of conditions often rivaling the synthetic fungicides (Cunningham, 2010; Janisiewicz and Conway, 2011). Thus, the combination of a moderate level of resistance in the fruit to postharvest decays with biocontrol agents or other alternatives may tilt the balance against the pathogens and result in adequate control of decay. Resistance in apples to various field diseases and insects has been explored in breeding programs; however, this is not the case for postharvest diseases (Volk et al., 2015). The apple gene pool has encountered a genetic bottleneck

\* Corresponding author. Fax: +1 304 728 2340.

E-mail address: [Wojciech.Janisiewicz@ars.usda.gov](mailto:Wojciech.Janisiewicz@ars.usda.gov) (W.J. Janisiewicz).

following centuries of domestication. Currently cultivated apples have practically no resistance to fungi causing fruit decay as breeders seldom evaluate their crosses for resistance to postharvest diseases (Ahmadi-Afzadi et al., 2013; Cornille et al., 2012). In the late 1980 to mid-1990 the USDA supported four expeditions to Central Asia including Kazakhstan to collect wild *Malus sieversii*, the progenitor of the cultivated apple *Malus domestica* (Forsline et al., 2003; Volk et al., 2015). This resulted in establishing a collection of these accessions at the Plant Genetic Research Unit in Geneva, NY as a part of the USDA-ARS National Plant Germplasm System. The entire collection includes many accessions from Central Asia and other parts of the world with great variation in many horticultural traits (Dzhangaliev, 2003; Janick, 2003; Volk et al., 2015). The wild apples from the *M. sieversii* Kazakhstan collection and from several other species obtained from Central Asia and Europe, were evaluated for resistance to *Penicillium expansum* and *Colletotrichum acutatum* and several immune and resistant accessions were discovered (Janisiewicz et al., 2008; Jurick et al., 2011). These accessions can be exploited in breeding programs for resistance against pathogens causing fruit decays after harvest. Most postharvest decays of apples originate from the infection of wounds made during harvest and handling. Some pathogens, such as *Penicillium* spp., infect fruit exclusively through wounds. Thus, resistance of the apple fruit wounds to infection becomes of paramount importance. Knowing the mechanism(s) of the resistance in mature apples, especially at the wound site, can be very important in selecting accessions for breeding programs and evaluating future crosses.

There are a variety of resistance mechanisms reported to operate in harvested fruits (Johnson et al., 1998; Prusky, 2003). They can be constitutive or induced and can be manipulated by chemical, biological and/or physical treatments. The induction is generally transient and inadequate to use alone for commercial decay management in harvest mature fruit; however, the research in this area provides important information about the nature of the mechanism of resistance (Bi et al., 2007; Moscoso-Ramirez and Palou, 2013; Prusky, 2003; Quaglia et al., 2011; Sanzani et al., 2010; Spadoni et al., 2015; Wilson et al., 1994). In the case of cultivated apples, as the fruit ripens, resistance of the wounds to infection by *P. expansum*, *Botrytis cinerea* or *C. acutatum* declines quickly (Ahmadi-Afzadi et al., 2013; Buron-Moles et al., 2015a; Su et al., 2011; Vilanova et al., 2014a). This has not been the case with some recently evaluated *M. sieversii* apples from the Kazakhstan collection and several other wild apple species, where a high level of resistance and even immunity was detected in mature fruit (Janisiewicz et al., 2008; Jurick et al., 2011; Janisiewicz and Jurick, unpublished).

The decline in apple resistance associated with fruit maturation, often manifested by a decline in resistant wound responses, has been explained by: (a) increasing pH that causes the dissociation of benzoic acid to a non-toxic form in 'Bramleys' apples infected with *Gloeosporium perennans* and *Diaporthe pernicioso* (Brown and Swinburne, 1973; Swinburne, 1975); (b) declining buffering capacity to resist pH changes induced by "alkaline" (e.g., *C. acutatum*) and "acidic" (e.g., *P. expansum*) pathogens (Prusky et al., 2013); (c) a decline in inducible host-defense responses, especially in oxidative burst (production of H<sub>2</sub>O<sub>2</sub>), phenolics, and various enzymes including those from the phenylpropanoid pathway, as well as chitinases and B-1, 3 glucanases (Buron-Moles et al., 2015a,b; Schovankova and Opatova, 2011; Su et al., 2011; Torres et al., 2003). A histochemical study of the wound responses to *P. expansum* and *B. cinerea* infection in preclimacteric 'Golden Delicious' apples revealed thickening of the cell wall and an increase in the accumulation of phenolic substances, tannins, lignins and callose in the wounded tissue as time after wounding increased and was coupled with a decline

in rot development (Lakshminarayana et al., 1987). Lignin content, implicated in apple resistance to decay, was significantly higher in wounds of immature than mature 'Golden Smoothee' apples (Vilanova et al., 2012, 2014a), and was negatively correlated with the incidence of blue mold on 'Golden Delicious' apples (Valentines et al., 2005).

In order to explain the mechanisms involved in apple resistance to postharvest pathogens a comprehensive approach involving temporal and spatial regulation of the transcriptome, proteome and metabolome combined with histochemical and pathological analysis must be undertaken (Prusky et al., 2013). Significant strides have been made in determining the transcriptomic and proteomic factors that may lead to an explanation of the mechanism of resistance in cultivated apples (Buron-Moles et al., 2015a,b; Spadoni et al., 2015; Vilanova et al., 2014b), and in the genetic analysis of wild apples from the Kazakhstan collection (Norelli, 2013).

The main objectives of this study were to: (a) determine the dynamics of the resistance response in wounds of wild *Malus sieversii* apples from Kazakhstan and a two other species from England and Macedonia with various levels of resistance to *P. expansum*; and (b) determine the major histochemical changes in the wounded area during the resistance response to *P. expansum*.

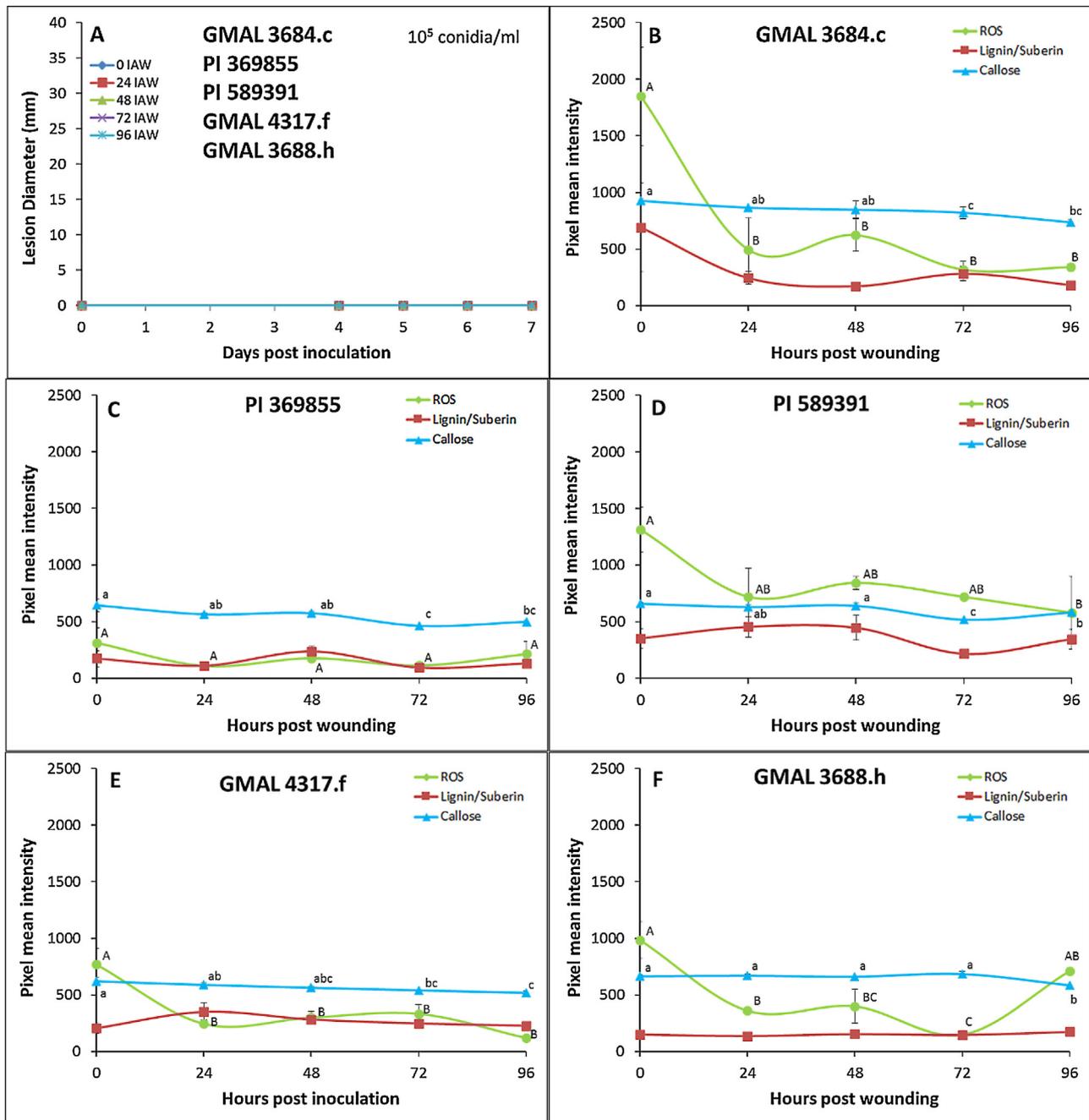
## 2. Materials and methods

### 2.1. Fruit

Apples were harvested at the USDA-ARS orchard in Geneva, NY, that maintains the apple germplasm collection (1), and brought to the USDA-ARS AFRS laboratory in Kearneysville, WV. Because conventional fruit maturity indices developed for commercial cultivars could not be applied to wild apples (Janisiewicz et al., 2008; Jurick et al., 2011) and frequently fruit with high starch content would drop off the trees within period of a few days, we decided to harvest fruit at the onset of formation of abscission layer (fruit starting to drop off the trees). This harvest time was related to a certain number of days after bloom. It was determined with close accuracy after a few years of observation for all accessions, except for PI 589391 and PI 3698553 that hung on the tree until the end of the growing season and were harvested just before the first frost. Apples from resistant/immune (GMAL 3684.c, GMAL 3688.h, GMAL 4317.f, PI 589391, and PI 369855), moderately resistant (GMAL 4304.d) and susceptible (GMAL 3623.i, and GMAL 4049.n, GMAL 4309.b) accessions were selected for the experiment (Janisiewicz et al., 2008; Jurick et al., 2011; Janisiewicz, unpublished). Accessions GMAL 3684.c was harvested 97 days post bloom (DPB), GMAL 4317.f at 115 DPB, GMAL 3623.i, GMAL 3688.h, GMAL 4049.n, GMAL 4304.d, and GMAL 4309.b at 130 DPB, and PI 589391, and PI 369855 ~150 DPB. The fruit were placed on fruitpack trays in plastic boxes and wounded within 24 h. In the replication of the experiment the fruit was stored for up to two months at 1 °C before it was subjected to wounding.

### 2.2. Pathogen

*P. expansum* isolate MD8, one of the most aggressive isolates in our collection, was originally isolated from a decayed apple and maintained on PDA (Janisiewicz, 1987). Conidia were collected from 7 to 10 day old cultures by adding 5 mL of 0.05% Tween 20 to the top of the culture. The conidial suspension was collected, vortexed for 30 s to disrupt conidial chains, centrifuged for 3 min at 13,793 × g, the pellet was resuspended in sterile distilled water. The conidia concentration was adjusted to 1 × 10<sup>5</sup> conidia/mL using a hemacytometer and a 1:10 dilution was made for the second suspension of 1 × 10<sup>4</sup> conidia/mL.

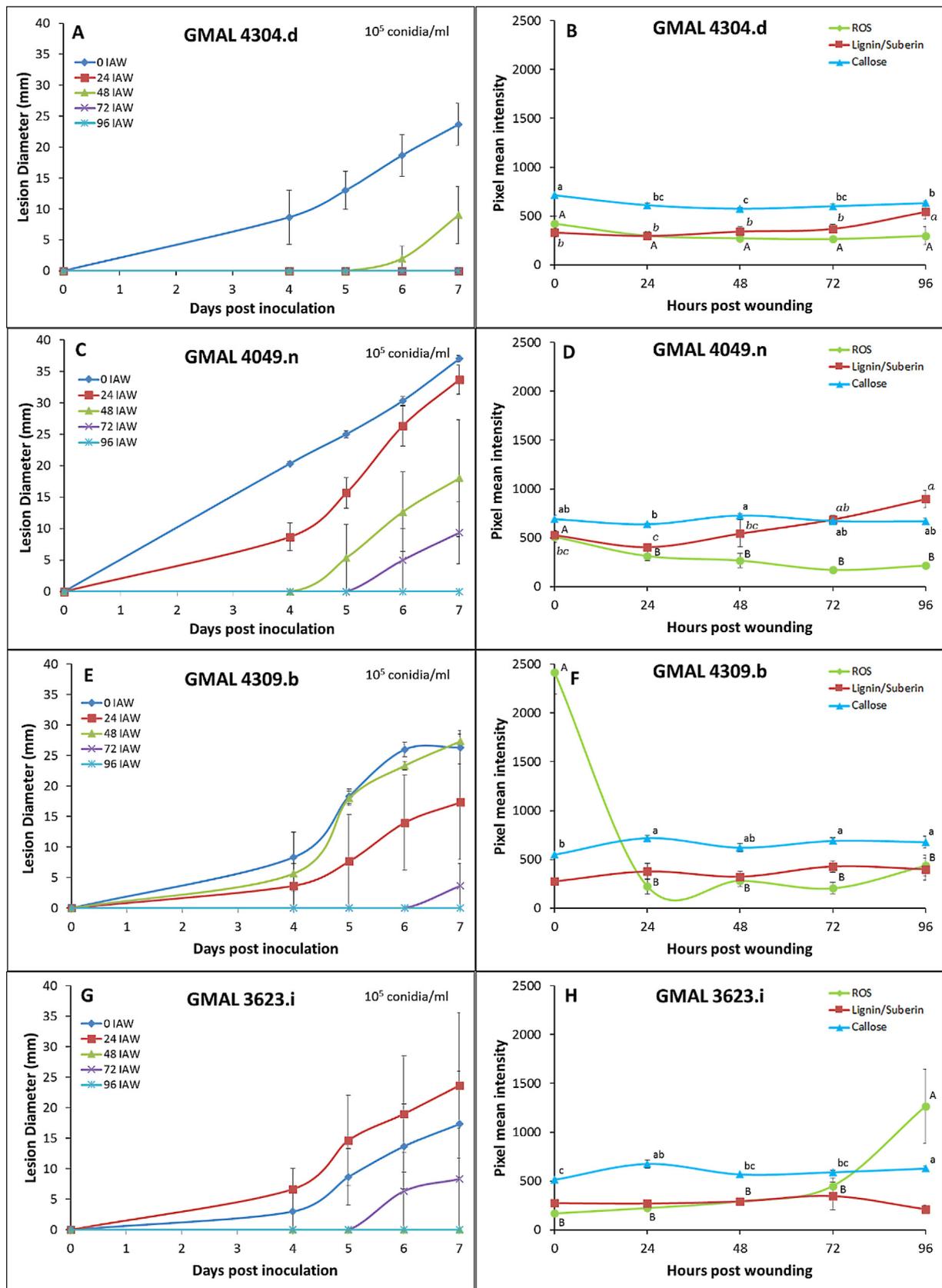


**Fig. 1.** Progression of decay development (A), and content of ROS, lignin/suberin, and callose (B–F) in resistant/immune wild apple accession from Kazakhstan and from England and Macedonia collections (PI accessions). For decay development, apples were inoculated with *Penicillium expansum* conidial suspensions at  $10^5$  conidia/mL at different times post wounding (0, 24, 48, 72, and 96 hpw) and incubated at 24 °C for up to 7 days post inoculation. The decay data was collected from 4 to 7 days after inoculation. No lesion has developed on any of the inoculated apples. For ROS, lignin/suberin and callose tests the wounded areas were sampled immediately after wounding and in 24 h intervals until 96 h post wounding (hpw), stained and observed under a fluorescent microscope where Pixel Mean Intensity (PMI) was recorded for the area to a distance of 1.86 mm from the edge of the wound into the flesh. Results from the wound inoculation and cytological tests are presented as means with  $\pm$  standard error of three replications. Means from the cytological tests were separated using LSD test ( $P=0.05$ ). For clarity, means for accessions where no significant differences in lignin/suberin occurred were not labelled.

### 2.3. Fruit wounding and inoculation

Fruit were wounded using a cylindrical wounding tool (3 mm deep  $\times$  3 mm dia.) and the tissue plugs were removed. All fruit were wounded at the same time with one wound per fruit at the mid-point between calyx and stem end. The wounds were inoculated with a suspension of *P. expansum* conidia immediately after wounding or 24, 48, 72 or 96 h after wounding. After inoculation, boxes were covered with lids and incubated at 24 °C

for 7 days. The severity (lesion expansion) of blue mold decay was determined daily beginning 4 days post inoculation (dpi) until the end of the experiment. For each inoculation, fresh *P. expansum* conidia were harvested from 7 to 10 day old cultures and the conidial suspensions were prepared as described above. Each fruit wound was inoculated with 25  $\mu$ L of the  $10^4$  or  $10^5$  conidia/mL suspension. There were three replications of 20 fruit for each treatment and the experiment was repeated.



**Fig. 2.** Progression of decay development (A, C, E, G) and content of ROS, lignin/suberin, and callose (B, D, F, H) in moderately resistant and susceptible wild apple accession from Kazakhstan. For decay development, apples were inoculated with *Penicillium expansum* conidial suspensions at  $10^5$  conidia/mL at different times post wounding (0, 24, 48, 72, and 96 hpw) and incubated at 24 °C for up to 7 days post inoculation. The decay data was collected from 4 to day 7 days after inoculation. For ROS, lignin/suberin and callose tests the wounded areas were sampled immediately after wounding and in 24 h intervals until 96 h post wounding (hpw), stained and observed under a fluorescent microscope where Pixel Mean Intensity (PMI) was recorded for the area at the distance of 1.86 mm from the edge of the wound into the flesh. Results from the wound inoculation and cytological tests are presented as means with  $\pm$  standard error of three replications. Means from the cytological tests were separated using LSD test ( $P=0.05$ ). For clarity, means for accessions where no significant differences in lignin/suberin occurred were not labelled.

## 2.4. Cytological changes

In conjunction with the inoculation test, a second set of apples was wounded, placed on cardboard fruit pack trays in plastic boxes and covered with lid. The wound areas 1 cm<sup>2</sup> with the wound in the center and to a depth of 1 cm were cut with a scalpel immediately after wounding and in 24 h intervals (0, 24, 48, 72, and 96 h) until 96 h. The wound area from each apple was hand sectioned to produce three sections for staining to detect the production of reactive oxygen species (ROS), and for deposition of callose, and lignin/suberin. The sections were made with a scalpel with No. 10 blade (Feather Safety Razor Co., Osaka, Japan) across the wound from the top to the bottom and were approximately 0.5 mm thick. There were three fruit for each of the five time points for each accession examined.

Determination of the production of ROS was obtained by staining for 10 min with 10 μM 2, 7-dichlorofluorescein diacetate (H<sub>2</sub>DCF-DA) (Sigma, St. Louis, MO), dissolved in DMSO and diluted in 50 mM MES buffer pH 6.5. Then, samples were washed two times in 50 mM MES and mounted on slides with 100–200 μL of fresh MES buffer for microscopic observation using the Zeiss Axio Zoom V16 Fluorescent Microscope System with the excitation at 470 nm, beamsplitter 495 nm (38HE filter cube) and emission at 525 nm. The intensity of the fluorescence was observed and the average intensity of the pixels was recorded for the area at a distance of 1.86 mm from the edge of the wound into the flesh.

Callose deposition was detected using aniline blue stain which was prepared as a 1% aqueous solution in sterile distilled deionized water (SDDW) from aniline blue powder (Sigma), filtered (0.22 μm) and stored in the dark. The wound sections were stained for 1 h, washed with SDDW for 10 min, mounted on glass slides and observed using Zeiss Axio Zoom V16 Fluorescent Microscope System with the excitation at 550 nm, beamsplitter 570 nm (43HE filter cube) and emission at 605 nm. The intensity of the fluorescence was observed and average intensity of the pixels for a standard area around the wound was recorded the same as above.

The hand sections of the fruit wound were stained with berberine (Brundrett et al., 1988) for lignin/suberin deposition. Berberine stain was prepared as a 0.1% w/v aqueous solution in SDDW using berberine hemisulfate salt (Sigma, St. Louis, MO) and filtered (0.22 μm). The staining procedure was the same as the aniline blue staining and the observation of fluorescent intensity was as described for ROS.

## 2.5. Statistical analysis

Results from the wound inoculation and cytological tests are presented as means with ± standard error of three replications. Results from the cytological tests were also analyzed using general linear model (GLM) of the Statistical Analysis System version 9.4 (SAS Institute Inc., Cary, NC, USA), and the last significant difference (LSD) test ( $P=0.05$ ) was used to separate means from the individual tests (ROS, callose, and lignin/suberin) for each accession.

## 3. Results and discussion

### 3.1. Wound resistance response

We realized from the onset of our investigations that the accessions from Kazakhstan and other parts of Central Asia were unusual regarding their phenological and physiological characteristics (Janisiewicz et al., 2008). Harvest dates were based on the onset of abscission layer development (fruit dropping off the tree).

Delaying harvest to the last possible moment provided assurance that the fruit was not harvested prematurely and that observed resistance was not related to the immaturity of the fruit. It was a compromise which may have resulted in missing some resistance that was lost as fruit matured on the tree. These variabilities and numerous instances of differential resistant responses of individual accessions to *P. expansum* and *C. acutatum* inoculation (Jurick et al., 2011) indicated a complexity of the resistance and a need to determine its durability before further (future) studies are undertaken to explain the basis for this resistance and its use in breeding programs.

Wound responses to infections have been a good estimator of resistance to postharvest decays in various harvested crops including pome fruits (Brown, 1989; Janisiewicz, 1988; Spotts et al., 1998; Su et al., 2011; Vilanova et al., 2014a,b). In general, the responses are much stronger in less mature fruit resulting in little or no decay, even after inoculation with high concentrations of pathogens, and decline as maturity progresses (Su et al., 2011; Torres et al., 2003; Vilanova et al., 2014a). Thus, it is important that the fruit are tested at proper (harvest) maturity. In our study, all immune accessions of the *M. sieversii* (GMAL 3684.c, GMAL 3688.h, GMAL 4317.f), *Malus × soulardii* (PI 589391), and *Malus sylvestris* (PI 369855) did not develop any lesions after inoculation of mature apples with either concentration, 10<sup>4</sup> or 10<sup>5</sup> conidia/mL, of *P. expansum* regardless of the time of inoculation after wounding (only results from the 10<sup>5</sup> are shown in Fig. 1A). Challenges with these two concentrations of the pathogen allowed for good separation of the wound resistant responses among accessions in the germplasm collection with a wide spectrum of resistance. It also indicates a high level of resistance in these accessions compared to currently used commercial cultivars where inoculation with a suspension of 10<sup>4</sup> conidia/mL overcame resistance, even on less mature more “resistant” ‘Golden Delicious’ apples (Torres et al., 2003). In addition, inoculum concentrations above 10<sup>5</sup> conidia/mL did not reduce onset of lesion development in ‘Golden Smoothie’ apples indicating a failure of the host resistance at this concentration (Vilanova et al., 2012). Also, immature ‘Golden Smoothie’ apples needed more than 72 h healing time to completely prevent decay development; however, on commercially mature (harvest mature) apples even after this healing period they developed small lesions (Vilanova et al., 2014a). In our “moderately resistant” accession (GMAL 4304.d) a very strong wound response occurred 24 h after wounding and only small lesions developed on fruit inoculated 48 h after wounding (Fig. 2A). Two susceptible accessions, GMAL 4049.n and GMAL 4309.b, required 96 h healing time before inoculation with the pathogen to develop a wound response that prevented decay development (Fig. 2C, E). The other susceptible accession GMAL 3623.i had somewhat similar responses; except that an additional strong wound response occurred after 48 h (Fig. 2G). These results indicate that the resistance in the five resistant accessions of wild apples is durable and will likely be maintained when they encounter similar inoculum loads under commercial conditions.

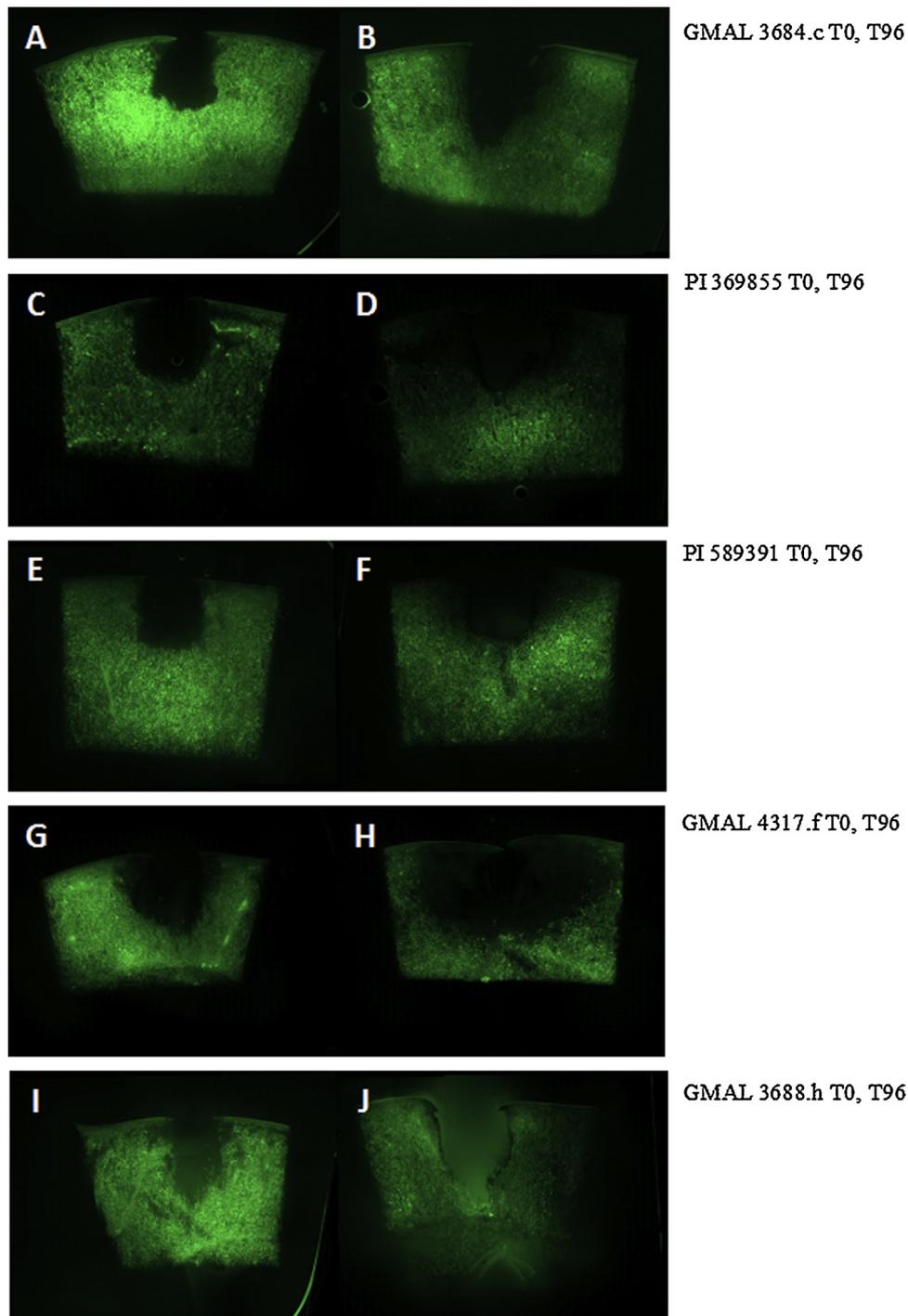
### 3.2. Cytological wound responses

The involvement of reactive oxygen species (ROS) in the resistance of apple to decays has been suggested for some time (Buron-Moles et al., 2015a,b; Castoria et al., 2003; Su et al., 2011; Torres et al., 2003). Various roles have been ascribed to ROS in plants including acting as a signaling molecule that activates various genes involved in resistant reactions, mediating senescence of the fruits, directly killing or inhibiting pathogens, and creating barriers by strengthening the cell wall by an oxidative processes involved in the formation of lignin and suberin (Tian et al., 2013; Wu et al., 1997). Studies in harvested apples have been

focused on ROS production in response to wounding and invasion of pathogens because the most important pathogens causing postharvest decays, including *Penicillium* spp., invade through wounds. Factors such as stages of maturity, environmental conditions, stress, and cultivars all had significant effects on the production of ROS and susceptibility of the wound to infection (Shao et al., 2010; Su et al., 2011; Torres et al., 2003; Vilanova et al., 2014a). In some cases an increase in ROS may not correspond to an increase in the resistance of apple wounds to infection. For example, heat stress causes an increase in production of ROS in ‘Gala’ apples that was associated with increasing susceptibility to

decay, while no increase in ROS occurred in ‘Red Fuji’ that become more resistant to decay under the same conditions (Shao et al., 2010).

In our studies, the ROS production after wounding varied among accessions regardless of their wound resistance response (Figs. 1–4). For immune accessions, ROS declined from a very high level of 1835, 1248, 943, 755 Pixel Mean Intensity (PMI) for GMAL 3684.c, PI 589391, GMAL 3688.h and GMAL 4317.f, respectively, immediately after wounding to 481, 675, 359, 256 PMI, respectively, during the first 24 h post wounding (hpw) (Fig. 1B, D, E, F). A measurable but not statistically significant increase occurred



**Fig. 3.** Fluorescent images of tissue around the wound from immune apple accessions from Kazakhstan and from England and Macedonia (PI accessions) stained with 2,7-dichloro-dihydro-fluorescein diacetate for reactive oxygen species (ROS). (A, C, E, G, I) Tissue stained immediately after wounding; (B, D, F, H, J) Tissue stained 96 h after wounding.

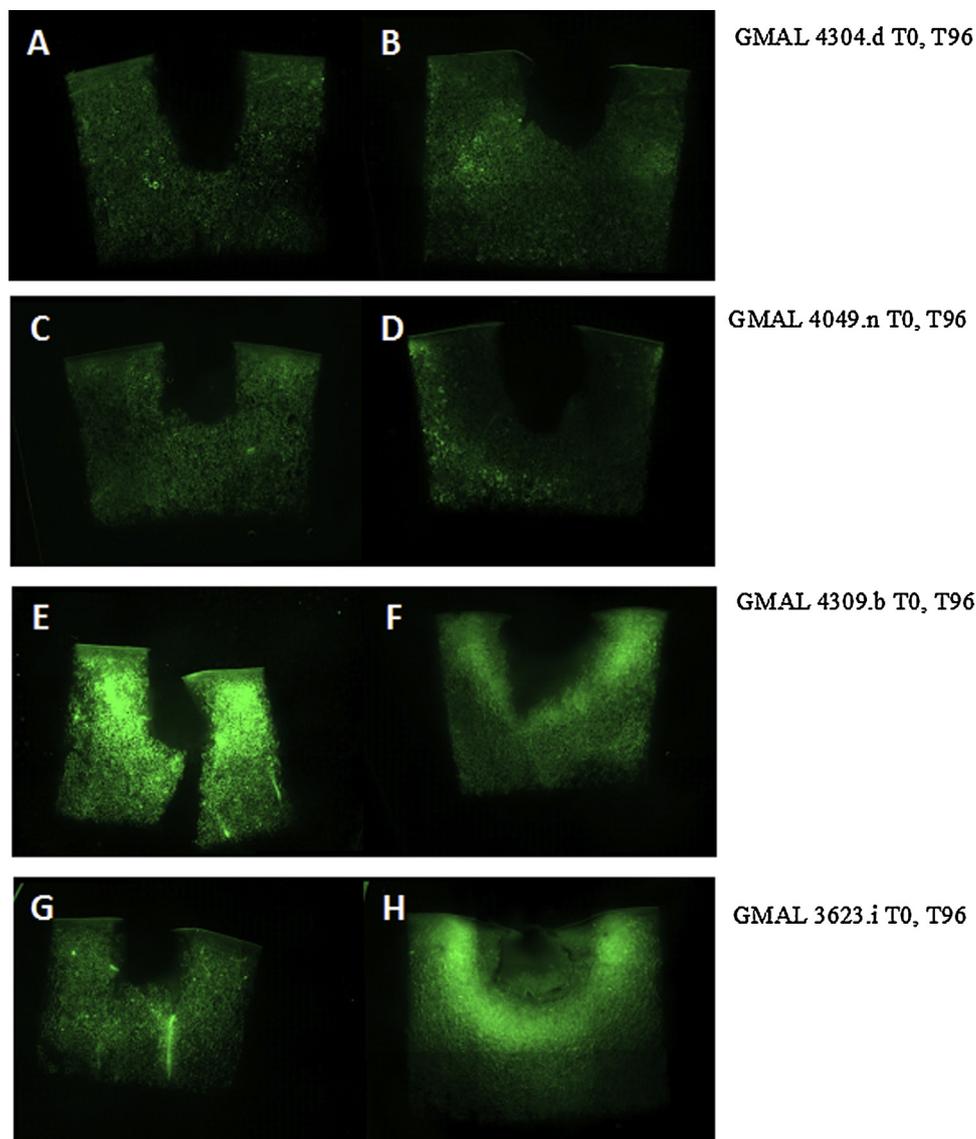
48 hpw followed by a slight decline in all of these accessions, but in the case of GMAL 3688.h, after further significant decline at 72 hpw, a significant increase to 718 PMI occurred at 96 hpw (Fig. 1F). In the immune accession PI 369855 the ROS production was consistently detected at very low level (Fig. 1C).

No increase in ROS was observed over the entire course of the experiment in accession GMAL 4304.d which had a very strong wound response (Fig. 2B). However, a high ROS intensity appears to be localized only in some cells scattered throughout the fruit tissue (Fig. 4A, B). A similar phenomenon was observed in one susceptible accession GMAL 4049.n, although these scattered cells were at a further distance from the wound area (Fig. 4C, D). Perhaps the most unusual case was observed with susceptible accession GMAL 4309.b where ROS intensity of 2420 PMI immediately after wounding was the highest of all the accessions tested (Fig. 2F). It declined rapidly to 222 PMI at 24 hpw and rose to 432 PMI at 96 hpw resulting in distinctly higher ROS intensity area around the wound (Fig. 4E, F). In the case of other susceptible accessions, GMAL 3623.i, an intensity of ROS of 168 PMI was the lowest of all the accessions tested and increased to 1201 PMI at 96 hpw (Fig. 2H) resulting in

the strongest density of ROS around the wound area of all accessions at that time point (Fig. 4G, H).

In general, in resistant apples a slight decline in lignin/suberin occurred during the course of the experiments, with the exception of moderately resistant GMAL 4304.d, where a small increase was observed at 96 hpw (Fig. 2B). In susceptible apples the amount of lignin/suberin fluctuated slightly except GMAL 4049.n, where a gradual increase occurred between 24 and 96 hpw (Fig. 2D). This agrees with observations on immature, commercially mature, or over-mature 'Golden Smoothie' apples (Vilanova et al., 2014a), and observations in wound tissue of 'd'Anjou' pear fruit (Spotts et al., 1998) where no accumulation of lignin was observed during first 72 h after wounding.

No drastic changes in content of callose was observed in resistant accessions although a significant gradual decline occurred during the 96 h course of the experiments (Fig. 1). However, the immune GMAL 3684.c consistently had the highest content of callose of all accessions throughout the study (Fig. 1B). Slight variations in callose occurred in susceptible accessions but no specific patterns emerged (Fig. 2D, F, H).



**Fig. 4.** Fluorescent images of tissue around the wound from moderately resistant and susceptible wild apple accessions from Kazakhstan stained with 2,7-dichloro-dihydrofluorescein diacetate for reactive oxygen species (ROS). (A, C, E, G) Tissue stained immediately after wounding; (B, D, F, H) Tissue stained 96 h after wounding.

Although the results from lignin/suberin and callose production do not negate earlier observations that they may play some role in resistance on immature fruit (Lakshminarayana et al., 1987; Spotts et al., 1998; Vilanova et al., 2014a), the production of ROS indicates that resistance in the wild apples is very complex and multifaceted. The rapid decline of the high concentration of ROS in the resistant accessions may actually prevent oxidative damage, mitochondrial disfunction, and prevent host cell death. This may be advantageous in developing host (fruit) resistance response to necrotrophic pathogens such as *P. expansum*. On the other hand, a very high concentration of ROS immediately after wounding may have a detrimental effect on *P. expansum* by reducing conidia germination and “pathogenic ability” (Tian et al., 2013; Qin et al., 2007). Necrotrophs may also induce ROS accumulation to activate programmed cell death in the host resulting in the release of nutrients and disease development (Govrin et al., 2006). In addition, ROS may also have a signaling function affecting host resistance that further complicates the explanation of this resistance (Glazebrook, 2005; Torres, 2010).

#### 4. Conclusions

In accessions with rapid wound responses that prevented development of the apple decay 24 hpw, an induced mechanism of resistance appears to be of paramount importance. However, in accessions with a high level of resistance/immunity from the onset of wounding, and even on fruit after 10 months in air storage (Janisiewicz, personal observation), innate immunity may be responsible for the lack of decay development. Ahmadi-Afzadi et al. (2015) demonstrated that “partial resistance” to blue mold in cold tolerant apple cultivars in Scandinavia to great extent depends on the presence of polyphenolic compounds. A similar phenomenon may have occur in the resistant/immune wild apples; however, it would be expected that the concentration of these and perhaps some novel compounds will be at much higher level. Differences in susceptibility to infection by *P. expansum* and *C. acutatum* in some accessions (Jurick et al., 2011) is an additional indication that resistance in the wild apples to postharvest decays is multifaceted and much more complex than the “partial resistance” demonstrated in immature and mature apples of commercially used cultivars (Ahmadi-Afzadi et al., 2015; Tahir et al., 2015; Vilanova et al., 2014a). This presents additional challenges in explaining the basis of these resistance mechanisms and it also may create unique opportunities for exploring these resistance mechanisms in breeding programs to obtain decay resistant cultivars.

Current economic constraints necessitate reduction in the size of the wild apple collection in Geneva, NY, and maintenance of most accessions represented by a single seedling will have to be discontinued. Saving selected accessions with different mechanisms of resistance, that may be valuable in studying these mechanisms and eventually can be used in breeding programs becomes of vital importance (Volk et al., 2015). Thus, accessions from this study will be preserved in the permanent germplasm collection and will be available for future study.

#### Acknowledgements

We would like to acknowledge the help of Mr. Bill Srmack in maintaining the collection of wild apple plants and assistance in collecting fruit samples. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA; USDA is an equal opportunity provider and employer. This research was sponsored by USDA CRIS 8080-22000-009-00D.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.postharvbio.2015.12.004>.

#### References

- Ahmadi-Afzadi, M., Tahir, I., Nybom, H., 2013. Impact of harvesting time and fruit firmness on the tolerance to fungal storage diseases in an apple germplasm collection. *Postharvest Biol. Technol.* 82, 51–58.
- Ahmadi-Afzadi, M., Nybom, H., Ekholm, A., Tahir, I., Rumpunen, K., 2015. Biochemical contents of apple peel and flesh affect level of partial resistance to blue mold. *Postharvest Biol. Technol.* 110, 173–182.
- Be, Y., Li, Y., Ge, Y., 2007. Induced resistance in postharvest fruits and vegetables by chemicals and its mechanism. *Stewart Postharvest Rev.* 6 (7), 1–7.
- Brown, E., 1989. Host defense at the wound site on harvested crops. *Phytopathology* 79, 1381–1384.
- Brown, A.E., Swinburne, T.R., 1973. Factors affecting the accumulation of benzoic acid in Bramley's seedling apples infected with *Nectria galligena*. *Physiol. Plant Pathol.* 3, 91–99.
- Brundrett, M.C., Enstone, D.E., Peterson, C.A., 1988. A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissue. *Protoplasma* 146, 133–142.
- Buron-Moles, G., Torres, R., Teixido, N., Usall, J., Vilanova, L., Vinas, I., 2015a. Characterizations of H<sub>2</sub>O<sub>2</sub> production to study compatible and non-host pathogen interactions in orange and apple fruit at different maturity stages. *Postharvest Biol. Technol.* 99, 27–36.
- Buron-Moles, G., Wisniewski, M., Vinas, I., Teixido, N., Usall, J., Droby, S., Torres, R., 2015b. Characterizing the proteome and oxi-proteome of apple in response to host (*Penicillium expansum*) and non-host (*Penicillium digitatum*) pathogen. *J. Proteomics* 114, 136–151.
- Castoria, R., Caputo, L., De Curtis, F., De Cicco, V., 2003. Resistance of postharvest biocontrol yeasts to oxidative stress: a possible new mechanism of action. *Phytopathology* 93, 564–572.
- Cornille, A., Gladieux, P., Smulders, M.J.M., Roldán-Ruiz, I., Laurens, F., Le Cam, B., Nersesyian, A., Clavel, J., Olonova, M., Feugey, L., Gabrielyan, I., Zhang, X.-G., Tenailon, M.I., Giraud, T., 2012. New insight into the history of domesticated apple: secondary contribution of the European wild apple to the genome of cultivated varieties. *PLoS Genet.* 8 (5), e1002703.
- Cunningham, N.M., 2010. Combination of treatments to replace the use of conventional fungicides for the commercial control of postharvest diseases of citrus fruit. *Stewart Postharvest Rev.* 6 (8), 1–8.
- Dzhangaliev, A.D., 2003. The wild apple tree of Kazakhstan. *Hortic. Rev.* 29, 263–303.
- Forsline, P.F., Aldwinckle, H.S., Dickson, E.E., Luby, J.J., Hokanson, S.C., 2003. Collection, maintenance, characterization, and utilization of wild apples of Central Asia. *Hortic. Rev.* 29, 1–61.
- Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227.
- Govrin, E.M., Rachmilevitch, S., Tiwari, B.S., Solomon, M., Levine, A., 2006. An elicitor from *Botrytis cinerea* induces the hypersensitive response in *Arabidopsis thaliana* and other plants and promotes the gray mold disease. *Phytopathology* 96, 299–307.
- Wild apple and fruit trees of central Asia. In: Janick, J. (Ed.), *Horticultural Reviews*, vol. 29. John Wiley and Sons, Hoboken, New Jersey, pp. 2–12.
- Janisiewicz, W.J., 1987. Postharvest biological control of blue-mold on apples. *Phytopathology* 77, 481–485.
- Janisiewicz, W.J., 1988. Wound healing process in pear fruit as related to infection by *Botrytis cinerea* and *Penicillium expansum*. *APS Abstracts of Presentations*. A670. 1988 APS Annual Meeting, San Diego, CA, November 13–17, APS Press.
- Janisiewicz, W.J., Conway, W.S., 2011. Combining biological control with physical and chemical treatments to control fruit decays after harvest. *Stewart Postharvest Rev.* 6 (1), 1–16 (16).
- Janisiewicz, W.J., Korsten, L., 2001. Biological control of postharvest diseases of fruits. *Ann. Rev. Phytopathol.* 40, 411–441.
- Janisiewicz, W.J., Saftner, R.A., Conway, W.S., Forsline, P.F., 2008. Preliminary evaluation of apple germplasm from Kazakhstan for resistance to postharvest blue mold in fruit caused by *Penicillium expansum*. *HortScience* 43, 420–426.
- Johnson, G.I., Highley, E., Joyce, D.C., 1998. Disease resistance in fruit. *Proceeding of an International Workshop*, Chiang Mai, Thailand, 18–21 May, 1997, Canberra, ACIAR Proceedings No. 80. xii, pp. 233.
- Jurick II, W.M., Janisiewicz, W.J., Saftner, R.A., Vico, I., Gaskins, V.L., Park, E., Forsline, P.L., Fazio, G., Conway, W.S., 2011. Identification of wild apple germplasm (*Malus* spp.) accessions from Kazakhstan with resistance to the postharvest decay pathogens *Penicillium expansum* and *Colletotrichum acutatum*. *Plant Breed.* 130 (4), 481–486.
- Lakshminarayana, S., Sommer, N.F., Polito, V., Fortlage, R.J., 1987. Development of resistance to infection by *Botrytis cinerea*, and *Penicillium expansum* in wounds of mature apple fruit. *Phytopathology* 77, 1674–1678.
- Lurie, S., Fallik, E., Kline, J.D., Kozar, F., Kovacs, K., 1998. Postharvest heat treatment of apples to control San Jose scale (*Quadraspidiotus perniciosus* Comstock) and blue mold (*Penicillium expansum* Link) and maintain fruit firmness. *J. Am. Soc. Hortic. Sci.* 123, 110–114.

- Moscoco-Ramirez, P., Palou, L., 2013. Evaluation of postharvest treatments with chemical inducers to control green and blue molds on orange fruit. *Postharvest Biol. Technol.* 85, 132–135.
- Norelli, J.L., 2013. Genetic analysis of *Malus sieversii* PI 613981 for resistance to postharvest apple fruit decay caused by *Penicillium expansum* (blue mold). Plant and Animal Genome XXI Conference (Abstr. PO418).
- Prusky, D., 2003. Mechanisms of resistance of fruits and vegetables to postharvest diseases. In: Bartz, J.A., Brecht, J.K. (Eds.), *Postharvest Physiology and Pathology of Vegetables*. Second ed. Marcel Dekker, Inc., New York, pp. 581–598 Revised and Expanded.
- Prusky, D., Alkan, N., Mengiste, T., Fluhr, R., 2013. Quiescent and necrotrophic lifestyle choices during postharvest disease development. *Annu. Rev. Phytopathol.* 51, 155–176.
- Qin, G.Z., Tian, S.P., Chan, Z.L., Li, B.Q., 2007. Crucial role of antioxidant proteins and hydrolytic enzymes in pathogenicity of *Penicillium expansum*: analysis based on proteomic approach. *Mol. Cell Proteomics* 6, 425–438.
- Quaglia, M., Ederli, L., Pasqualini, S., Zizzerini, A., 2011. Biological control agents and chemical inducers of resistance for postharvest control of *Penicillium expansum* Link. on apple fruit. *Postharvest Biol. Technol.* 59, 307–315.
- Ramanazzi, G., Lichter, A., Galber, F.M., Smilanick, J.L., 2012. Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. *Postharvest Biol. Technol.* 63, 141–147.
- Sanzani, S.M., Schena, L., De Girolamo, A., Ippolito, A., González-Candelas, L., 2010. Characterization of genes associated to induced resistance against *Penicillium expansum* in apple fruit treated with quercetin. *Postharvest Biol. Technol.* 56, 1–11.
- Schovankova, J., Opatova, H., 2011. Changes in phenols composition and activity of phenylalanine-ammonia lyase in apples after fungal infections. *Hortic. Sci. (Prague)* 38, 1–10.
- Shao, X., Tu, K., Tu, S., Su, J., Zhao, Y., 2010. Effect of heat treatment on wound healing in Gala and Red Fuji apple fruit. *J. Agric. Food Chem.* 58, 4303–4309.
- Soliva, F.R., Elez, F.P., Martin, O., 2014. Advances in non-traditional physical methods for postharvest management of fruits and vegetables. *Stewart Postharvest Rev.* 10 (6), 1–6.
- Spadoni, A., Guidarelli, M., Phillips, J., Mari, M., Wisniewski, M., 2015. Transcriptional profiling of apple fruit in response to heat treatment: involvement of a defense response during *Penicillium expansum* infection. *Postharvest Biol. Technol.* 101, 37–48.
- Spotts, R.A., Sanderson, P.G., Lennox, C.L., Sugar, D., Cervantes, L.A., 1998. Wounding, wound healing and staining of mature pear fruit. *Postharvest Biol. Technol.* 13, 27–36.
- Su, J., Tu, K., Cheng, L., Tu, S., Wang, M., Xu, H., Zhan, G., 2011. Wound-induced H<sub>2</sub>O<sub>2</sub> and resistance to *Botrytis cinerea* decline with ripening of apple fruit. *Postharvest Biol. Technol.* 62, 64–70.
- Swinburne, T.R., 1975. Microbial proteases as elicitors of benzoic acid accumulation in apples. *Phytopathol. Z.* 82, 152–162.
- Tahir, I., Nybom, H., Ahmadi-Afzadi, M., Roen, K., Sehic, J., Roen, D., 2015. Susceptibility to blue mold caused by *Penicillium expansum* in apple cultivars adapted to a cool climate. *Eur. J. Hortic. Sci.* 80, 117–127.
- Tian, S., Qin, G., Li, B., 2013. Reactive oxygen species involved in regulating fruit senescence and fungal pathogenicity. *Plant Mol. Biol.* 82, 593–602.
- Torres, M.A., 2010. ROS in biotic interactions. *Physiol. Plant* 138, 414–429.
- Torres, R., Valentines, M.C., Usall, J., Vinas, I., Larrigaudiere, C., 2003. Possible involvement of hydrogen peroxide in the development of resistance mechanism in 'Golden Delicious' apple fruit. *Postharvest Biol. Technol.* 27, 235–242.
- Valentines, M.C., Vilaplana, R., Torres, R., Usall, J., Larrigaudiere, C., 2005. Specific roles of enzymatic browning and lignification in apple disease resistance. *Postharvest Biol. Technol.* 36, 227–234.
- Vilanova, L., Teixido, N., Torres, R., Usall, J., Vinas, I., 2012. The infection capacity of *P. expansum* and *P. digitatum* on apples and histochemical analysis of host response. *Int. J. Food Microbiol.* 157, 360–367.
- Vilanova, L., Vinas, I., Torres, R., Usall, J., Buron-Moles, G., Teixido, N., 2014a. Increasing maturity reduces wound response lignification processes against *Penicillium expansum* (pathogen) and *Penicillium digitatum* (non-host pathogen) infection in apples. *Postharvest Biol. Technol.* 88, 54–60.
- Vilanova, L., Wisniewski, M., Norelli, J., Vinas, I., Torres, R., Usall, J., Phillips, J., Droby, S., Teixido, N., 2014b. Transcriptomic profiling of apple in response to inoculation with a pathogen (*Penicillium expansum*) and non-pathogen (*Penicillium digitatum*). *Plant Mol. Biol. Rep.* 31, 566–583.
- Volk, G.M.C., Chao, T., Norelli, J., Brown, S.K., Fazio, G., Peace, C., McFerson, J., Zhong, G.-Y., Bretting, P., 2015. The vulnerability of US apple (*Malus*) genetic resources. *Genet. Resour. Crop Evol.* 62, 765–794.
- Wilson, C.L., El Ghaouth, A., Chalutz, E., Droby, S., Stevens, C., Lu, J.Y., Khan, V., Arul, J., 1994. Potential of induced resistance to control postharvest diseases of fruits and vegetables. *Plant Dis.* 78, 837–844.
- Wu, G., Shortt, B.J., Lawrence, E.B., Leon, J., Fitzsimmons, K.C., Levine, E.B., Raskin, I., Shah, D.M., 1997. Activation of host defense mechanism by elevated production of H<sub>2</sub>O<sub>2</sub> in transgenic plants. *Plant Physiol.* 115, 427–435.