Chapter 2

Real time monitoring of ethylene during fungal-plant interaction by laser-based photoacoustic spectroscopy

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INTRODUCTION

The quality of agricultural products at the time they arrive at the consumers strongly depends on the developmental stage at harvest, shipping and storage conditions. It is commercially advantageous that fruit and vegetables have a long shelf life and do not deteriorate immediately after harvest. In many deteriorative processes the plant hormone ethylene plays an important role; by controlling the ethylene production or sensitivity important benefits can be obtained (Saltveit, 1999).

The plant hormone ethylene

Ethylene is involved in virtually all aspects of the plant life cycle, as well as in the plants response to many environmental stimuli. In the broadest of terms, ethylene is responsible for signaling changes during germination, growth, flower and fruit development, senescence of plant organs, programmed cell death, the onset of plant defense mechanisms and the action of other plant hormones. Biotic stress (e.g. pathogen attack) and abiotic stress conditions (e.g. wounding, hypoxia, ozone, chilling, and freezing) elicit ethylene synthesis in plants (Abeles, 1992; Mattoo and Suttle, 1991).

The elucidation of the ethylene biosynthetic pathway and the molecular cloning of genes encoding the enzymes involved have provided insight into the regulation of ethylene biosynthesis in plants. Plants biosynthesize ethylene via the Yang cycle, wherein methionine is converted to S- adenosylmethionine (SAM) by the enzyme SAM synthase. The conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) is then catalyzed by the enzyme ACC synthase (ACS). ACC is oxidized to ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984) (Fig. 1). The conversion of SAM to ACC is generally considered to be the rate-limiting step in the synthesis of ethylene (Kende, 1993). Both latter enzymes play a role in the regulation of ethylene biosynthesis and are encoded by small gene families.

Ethylene biosynthesis in microorganisms

In addition to plants, some microorganisms, including phytopathogenic fungi and bacteria, can synthesize ethylene themselves. Except for few fungal species, such as the slime mold *Dictyostelium mucoroides* (Amagai and Maeda, 1992) and *Penicillium citrinum* (Jia *et al*., 1999), the ACC pathway for ethylene biosynthesis has not been found operative in microorganisms. Presently, two different ethylene biosynthetic pathways have been established in microorganisms (Fukuda *et al*., 1993). Ethylene can be produced either from glutamic acid via 2-oxoglutarate as in *Penicillium digitatum* (Fukuda *et al*., 1989a) and in *Pseudomonas syringae* (Nagahama *et al*., 1991) or from methionine via 2-keto-4-methylbutyric acid (KMBA) as in *Escherichia coli* (Ince and Knowles, 1986), *Cryptococcus albidus* (Fukuda *et al*., 1989b),

Colletotrichum musae (Daundasekera *et al*., 2003) and in *Botrytis cinerea* (Cristescu *et al*., 2002; Chague *et al*., 2002) (Fig. 1). Additionally, KMBA has been identified as an intermediate in methionine-derived ethylene biosynthesis by microbial cultures in soil (Nazli *et al*., 2003).

Effect of ethylene on fungal development

It was reported that ethylene has different effects on various phases of fungal development *in vitro.* Exogenous application of ethylene stimulates conidial germination of *B. cinerea*, *Penicillium expansum, Rhizopus stolonifer* and *Gloeosporium perennans* (Kepczynski and Kepczynska, 1977), *P. digitatum, P. italicum, Thielaviopsis paradoxa (*El-Kazzaz *et al*., 1983), *Diplodia natalonis* and *Phomopsis citri* (Abeles, 1973). Elad (2002) showed that ethylene did not affect conidial germination and hyphal growth of *B. cinerea* on PDA media (potato dextrose agar), whereas on glass, tomato or bean leaf surfaces both germination rate and germ tube elongation were enhanced. A specific inhibitor of ethylene action in plants, 2,5-norbornadiene (NBD) inhibited growth of hyphae and mycelium and retarded the *B. cinerea* development (Kepczynska, 1989; 1993). A similar inhibitory effect was reported following application of the plant ethylene production inhibitor, aminoethoxyvinylglycine –AVG- a specific inhibitor of ACC synthase (Fig. 1), which reduced mycelium growth and sporulation of *B. cinerea*. As ethylene biosynthesis in *B. cinerea* does not involve ACC synthase, the target of AVG is probably some other aminotransferase and the effect may not related to ethylene biosynthesis.

Many fungi are known to remain dormant at the fruit surface until the fruit ripens, at which time the fungus infects the fruit. In some fungi ethylene was found to play a role as a signaling molecule. For instance, in *Colletotrichum gloeosporoides* and *C. musae* that attack ripe fruit, exposure to ethylene induces germination and appressorium formation. The reception of ethylene by the fungus was supposed to act through a mechanism with similarity to the receptor-mediated effects of ethylene in plants. Sensing of ethylene was blocked by the ethylene perception inhibitors, silver thiosulphate (STS) and 2,5-norbornadiene (NBD), while the ethylene analog propylene (but not methane) could substitute for ethylene. On transgenic tomato fruits, that did not produce ethylene, the fungus was unable to germinate. Upon treatment with ethylene the spores germinated and produced multiple appressoria and infection lesions (Kolattukudy *et al*., 1995).

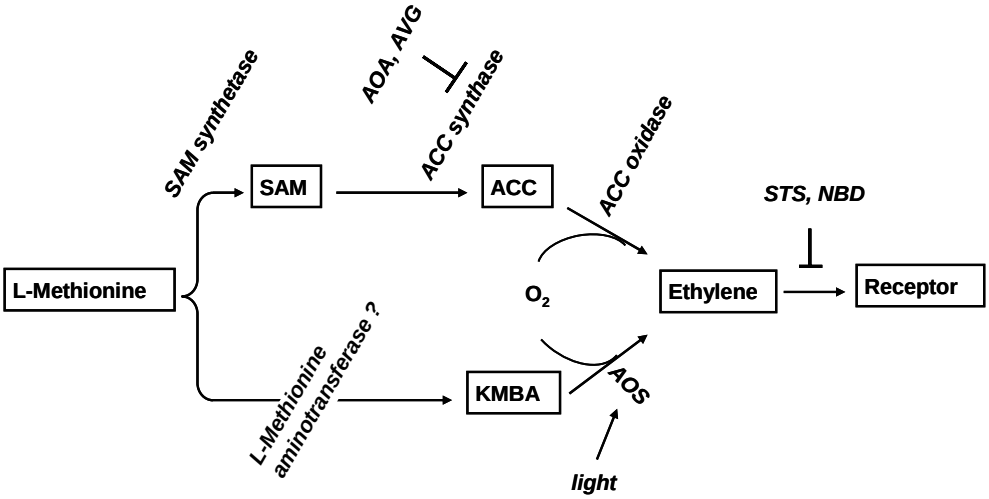

Figure 1. Ethylene biosynthesis in plants and *B. cinerea.*

In *Aspergillus parasiticus* ethylene was found to affect fungal development and aflatoxin synthesis in a dose dependent manner. The effect of ethylene could be influenced by treatment with the plant ethylene binding inhibitor 1-MCP (Roze *et al*., 2004). Such observations suggest that fungi can sense ethylene by a similar mechanism as plants. In plants ethylene is sensed by two component histidine kinases (Chang and Stadler, 2001) and recently, similar type of proteins was identified in *B. cinerea* (Catlett *et al*., 2003). However, the functionality of these proteins has not yet been elucidated.

Host-pathogen interactions and ethylene

Enhanced ethylene production is one of the earliest responses of plants to the perception of a pathogen (Boller, 1991) and ethylene has been associated with both resistance and susceptibility to disease (Knoester *et al*., 1998). A survey of the literature yields conflicting reports on the role of ethylene in pathogenesis (Johnson and Ecker, 1998). Ethylene may be a stimulus for defense responses by activating the plant defense genes which thereby lead to increasing plant resistance or, conversely, it may play a role in disease symptom development and in the breakdown of internal plant resistance (Boller, 1991; Abeles *et al*., 1992; Lund *et al*., 1998). Depending of the type of pathogen and plant species, the role of ethylene can be dramatically different. This diversity can be explained by taking into account the involvement of ethylene in the multiple physiological processes in plants and its interaction with other hormones and pathways. External application of ethylene reduces, increases or does not have any effect on disease incidence depending on the plant-pathogen system (El-Kazzaz *et al*., 1983; Elad, 1990; Marte *et al*., 1993).

Ethylene promotes fruit ripening, induces necrosis and chlorosis and accelerates the senescence in plants (Matoo and Suttle, 1991; Abeles *et al*., 1992). Ripe fruits and senescent or wounded plant organs are more susceptible to *B. cinerea* infection. Increased ethylene production is also characteristic of the hypersensitive response (HR) during the incompatible combination of an avirulent pathogen and its resistant host in which cells at the infection site die to form a necrotic lesion. As a result, a biotrophic pathogen is deprived of nutrients and its growth restricted (Cohn *et al*., 2001). On the contrary, some so-called necrotrophic pathogens, such as *B. cinerea,* may benefit from HR by using the dead tissues as a food support to further spread and invade healthy living tissues (Govrin and Levine, 2000).

Different defense mechanisms are involved in resistance and each of them has the capacity to withstand infection of certain pathogens (Thomma *et al*., 2001). The plant defense-related processes activated by ethylene include production of pathogenesis-related (PR) proteins (Rodrigo *et al*., 1993, van Kan *et al*., 1995), production of phytoalexins and lignin biosynthesis (Fan *et al*., 2000), activation of hydrolases (Boller, 1988, Brown and Lee, 1993), the induction of the phenylpropanoid pathway (Chappell *et al*., 1984) and cell wall alterations (Bell, 1981). Although the synthesis of the pathogenesis-related proteins can also be induced by ethylene-independent pathways (Dixon and Lamb, 1990), biological elicitation of some of them may require a functional ethylene response (Penninckx, *et al*., 1996). However, enhanced endogenous ethylene production is not always a requirement for the induction of defense responses (Boller, 1991; Bent *et al*., 1992; Ciardi *et al*., 2000; Lawton *et al*., 1995).

Van Loon (1984) suggested that a large part of the plant damage during pathogen infection is caused by autocatalytic ethylene synthesis and not from the direct action of the pathogen. Based on this, it was proposed that exogenous ethylene often increases the disease severity and, moreover, that inhibitors of ethylene synthesis may decrease the fungal infection severity. There are many examples supporting this hypothesis: (i) Increase of ethylene production due to the infection has been correlated with increased plant disease susceptibility in the case of wheat plants infected with *Septoria nodorum* (Hyodo, 1991). (ii) Exogenous ethylene applied to cucumber plants prior to infection increased disease severity in the case of *Colletotrichum lagenarium* (Biles *et al*., 1990) and for *Verticillium* wilt of tomato (Cronshaw and Pegg, 1976). (iii) Specific inhibitors of ethylene synthesis reduced disease severity to *B. cinerea*

infection in rose and carnation flowers, detached leaves of tomato, pepper, French-bean and cucumber (Elad 1990 and 1993; Boller, 1991). (iv) Cotton plants pretreated with AVG (an inhibitor of ethylene biosynthesis) showed decreased disease severity when infected with *Alternaria* (Bashan, 1994). However, virtually conflicting results are observed with tomato plants pretreated with ethylene or the inhibitor of ethylene perception, 1-methylcyclopropene (MCP). Ethylene pretreatment caused a decreased susceptibility against *B. cinerea* and MCP pretreatment resulted in increased susceptibility (Diaz *et al*., 2002). These examples show that ethylene may play a different role in disease development depending on the time it is applied or produced with respect to the timing of infection. Ethylene can affect the disease development by its possible direct action on the pathogen and/or indirectly by inducing various modifications in host plant metabolism (Kader, 1985).

Ethylene was found to stimulate fungal growth directly (Brown and Lee, 1993), but also to increase the activity of certain abscission-associated enzymes in the plant and, therefore, to predispose the plant to pathogen invasion (Brown and Burns, 1998). Although it was reported by El-Kazzaz *et al*., (1983) that ethylene stimulates the growth of *B. cinerea* and *P. italicum*, Palou *et al*., (2003) found that ethylene did not affect aerial mycelial growth on table grapes infected with *B. cinerea*. Neither incidence, nor disease severity to *Monilinia fructicola* of stone fruit was affected by exogenous application of ethylene, suggesting that ethylene plays no role in the pathogenicity of this fungus.

An additional approach to study the effect of ethylene on disease development is with ethylene-insensitive mutants (Knoester *et al*., 1998; Geraats *et al*., 2002; Hoffman *et al*., 1999). For example, the ethylene-insensitive *Arabidopsis thaliana* mutant *ein2* displayed enhanced susceptibility to *B. cinerea* (Thomma *et al*., 1999), but decreased susceptibility to infection with the beet cyst nematode (Wubben *et al*., 2001). Soybean mutants with reduced ethylene sensitivity developed more severe symptoms than the wild type when infected by necrotrophic pathogens, *Septoria glycines* and *Rhizoctonia solani*, and less severe chlorotic symptoms when infected by biotrophic pathogens, *P. syringae* pv. *glycines* and *P. sojae* (Hoffman *et al*., 1999). Ethylene-insensitive *A. thaliana* and tomato lines did not display high susceptibility to the bacteria *Pseudomonas* and *Xenathomonas* or the fungal species *Fusarium* and the Oomycete *Peronospora* (Bent, *et al*., 1992; Lawton *et al*., 1995; Lund *et al*., 1998). It was found that ethylene-insensitive tobacco plants Tetr 1 (Tetr 1, expressing the mutant *A. thaliana etr1- 1*gene) were also more susceptible than the wild type plants to several other fungi, including *Colletotrichum destructivum* (Chen *et al*., 2003) and *Chalara elegans* (Knoester *et al*., 1998; Geraats *et al*., 2002). Plants that did not produce ethylene developed much larger necrotic areas, while addition of ethylene restricted disease spreading. However, high levels of ethylene are usually destructive to plant growth and health and facilitate their damage by pathogen. During the fungal-plant interaction both the partners can produce ethylene, usually via a different pathway. Since methionine is the common precursor for ethylene biosynthesis in plants and in different microorganisms, it is difficult to determine if ethylene originates from the plant or the fungus. Usually, ethylene production by fungi was studied under *in vitro* conditions and then correlated with the ethylene emission from the infected host. Currently, there is no evidence of ethylene production by a specific fungus *in planta* and it is not known if fungal ethylene may play a role in triggering host ethylene production. Molecular tools to study the biology of fungi (ten Have *et al*., 2001; Wubben *et al*., 2000) will enable the role of fungus-produced ethylene in pathogenesis and the isolation of the genes involved in fungal ethylene biosynthesis and their deletion will unequivocally show whether ethylene production by the fungus plays a role in the fungus-plant interaction. In our research group we have developed a new approach to study the pathogen-host interaction by using a laser spectroscopic technique. As application, we chose the tomato fruit - *B. cinerea* system. *B. cinerea* is an important worldwide pathogen that attacks more than 200 plant species and

causes extensive crop losses to many field-grown and greenhouse crops. It is visible as the well-known grey mold on fruits, vegetables, ornamentals, trees, shrubs and various types of foods. *B. cinerea* infects many plant species, but also various organs at different developmental stages of a particular plant host. Fruit and stem rot, blossom blight, stem cankers, leaf spots, bulb-, corm-, tuber- and root-rots and twig blight are all manifestations of the fungus in plants. Under humid conditions it can cause massive losses in yield and quality, particularly in wine and fruit production. The losses imposed by this pathogen require the intense use of fungicides worth about € 50-100 million per year in Europe. *B. cinerea* is one of the most ubiquitous and serious fungal diseases of greenhouse tomatoes. Even more research was done on the *B. cinerea* infection of the vegetative plant parts (stem and leaves) than on fruits. It has been shown that *B. cinerea* produces ethylene *in vitro* most probably via pathway for ethylene formation using KMBA (Cristescu *et al*., 2002; Chague *et al*., 2002). Therefore, analysis of ethylene emission from the plant-pathogen system, such as tomato-*B. cinerea* becomes a complex phenomenon and more information is required to elucidate the contribution of each organism to the total ethylene produced. We present a laser-based ethylene detector suitable to monitor on-line the ethylene released during the infection process. The instrument allows ethylene emission in a flow-through system with a detection limit down to 10 pptv (pptv = parts-per-trillion volume, $1{:}10^{12}$) (Bijnen *et al*., 1996) and has relatively high time resolution for measuring the dynamics of ethylene production by *B. cinerea in vitro* and by infected tomatoes. We indicate it as a powerful tool to study the relationship between ethylene released by the fungus *in vitro* and the enhanced ethylene production in *B. cinerea* infected tomato with respect to disease development. Especially for this particular type of interaction when both, the host and the pathogen, are able to produce ethylene, it is difficult to separate their contribution to the total ethylene emission by the host-pathogen system. In combination with an effective broad range of well characterised chemical inhibitors and pathway intermediates related to ethylene biosynthesis, the use of this technique enables us to produce a comprehensive description of the mode of ethylene formation and action in *B. cinerea*, both *in vitro* and *in vivo*.

Real time monitoring of ethylene

To follow dynamic processes in plants, it is necessary to measure ethylene directly and with high resolution in time. This can be achieved if a flow-through system in line with sampling cuvettes is combined with the extremely sensitive laser-based photoacoustic detector. The schematic diagram of the set up is presented in Fig. 2. The various parts of the system are computer controlled, enabling a fully automated sampling of ethylene production rates of biological tissue for periods up to several weeks. The detector consists of a line-tunable CO_2 laser which emits radiation in the 9-11 μm infrared wavelength region and a photoacoustic cell, through which the laser light is directed for detecting the gas of interest (Harren and Reuss, 1997; te Lintel Hekkert *et al*., 1998). The laser-based ethylene detector is able to distinguish between different gases by making use of their wavelength dependent "fingerprint" absorption. Thanks to its distinct fingerprint-like spectrum in the CO_2 laser wavelength range (Brewer *et al*., 1982), ethylene can be measured with very high sensitivity, exhibiting a detection limit of three orders of magnitude better than gas chromatography (i.e. 10 $pL \cdot L^{-1}$).

Since the last decade we have used the laser-based photoacoustic systems to determine on-line ethylene release in various processes in plants and microorganisms, such as seed germination (Petruzzelli *et al*., 1995; Thuring *et al*., 1994), flower senescence (Woltering *et al*., 1993; Wagstaff *et al*., 2005), diffusion through aerenchymatous roots (Visser *et al*., 1997), submergence (Voesenek *et al*., 1993), fruit ripening (de Vries *et al*., 1995, 1996), nitrogen fixation by cyanobacteria (Zuckermann *et al*., 1997a; Staal *et al*., 2001, 2003), interaction with auxin (van der Bussche *et al*., 2003), dehydration (Leprince *et al*., 2000) and circadian rhythm (Thain *et al*, 2004).

Trace gases released by the biological samples (plated fungi or infected fruits) are transported to the photoacoustic cell through a flow system using air as carrier gas. Once inside the photoacoustic cell, traces of ethylene absorb the laser radiation and convert it into heat which will further generate an increase of pressure inside a closed volume. By modulating the laser beam with a chopper, pressure waves (i.e. sound) are generated and detected with a sensitive miniature microphone. The amplitude of the acoustic waves is directly proportional to the concentration of ethylene in the photoacoustic cell.

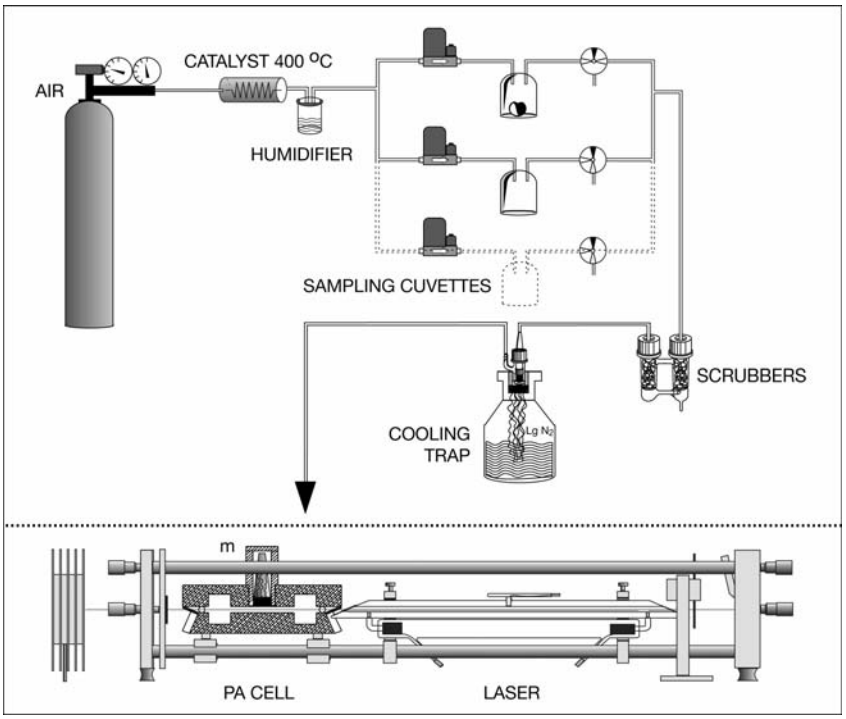

Figure 2. The ethylene detection set-up. A) gas flow system. B) laser-based ethylene detector consisting of a photoacoustic (PA) cell and a CO_2 laser. Ethylene released by the biological samples (plated fungi or infected fruits) is transported to the PA cell where it absorbs the laser radiation and gives rise to an acoustic wave. The amplitude of this wave is measured with a sensitive microphone (m) and is directly proportional with the ethylene concentration.

The overall measuring sequence can be chosen in function of the dynamics of the process under study. It can vary from less than 10 seconds for fast processes up to about one minute.

The gas flow through the measuring system can be controlled using electrical three-way valves that switch a particular gas stream to the photoacoustic cell (on-position) or into the laboratory (off-position). In this way the gas emission from a number of cuvettes (up to 8 per experiment) containing the biological samples can be transported to the photoacoustic cell alternately and at controlled flow rates. The flow is continuously monitored and adjusted by mass flow controllers.

Other interfering gases released by the samples might influence the quantification of ethylene emission, due to the overlap between their spectral absorption and the CO_2 laser wavelengths; therefore, a number of scrubbers and traps are introduced in the measuring system to remove them from the gas flow. A platinum based catalyzer (platinum on Al_2O_3)

which operates at minimum 400°C placed before the entrance of the cuvettes provides air free of any traces of ethylene (or other hydrocarbons). When monitoring low ethylene concentrations, the CO_2 and water concentrations have to be reduced before entering the photoacoustic cell. Consequently, a scrubber with KOH (moist pellets) is usually used to reduce the CO_2 concentration below 1 ppmv and a tube with $CaCl_2$ (granules) is placed directly after it in order to decrease the water content in the gas flow. Ethanol and some other heavier hydrocarbons are removed by inserting a cooling trap (-150°C) into the gas flow system just before the photoacoustic cell. In addition, the gas flow was filtered by passing through 0.2 μm millipore filters placed at the inlet and outlet of the sampling cuvettes.

From the obtained emission rates, readings of an empty cuvette are subtracted in order to adjust for externally induced variations (e.g. over hours or days). In most of the cases, the ethylene production from the fungi was related to the emission rate by multiplying the measured value with the flow rate, and expressed in nl h^{-1}. While monitoring the emission by fruits, the rate of ethylene production was expressed in nl h^{-1} g^{-1}fresh weight.

ETHYLENE PRODUCTION AND PERCEPTION BY *BOTRYTIS CINEREA IN VITRO*

Ethylene production by *B. cinerea* in vitro

When plants mature and die, the senescing tissues serve as a food base for many microorganisms. *B. cinerea* is such an organism. This fungus very quickly colonizes wounded, dead or dying stems, leaves, flowers, and fruits. As a necrotrophic pathogen, *B. cinerea* has the ability to kill and macerate the host cells before invading them to obtain the nutrients for its growth. By this mechanism, using the previously colonized dead tissues as a food support, the fungus can spread and invade healthy living tissues (Jarvis, 1977).

The ability of *B. cinerea* to adapt to various environmental conditions has been investigated and different mechanisms of its action in function of the attacked host tissue were proposed (Barkai-Golan *et al*., 1988; Elad and Eversen, 1995; ten Have *et al*., 2001; von Tiedemann, 1997; Yang and Hoffman, 1984). However, there is no broad understanding of the "attack strategies" of this fungus. One intriguing part in this scenario is the role of ethylene. Ethylene is generated during the *Botrytis*-host interaction possibly by both organisms as both plants and *B. cinerea* have the ability to produce it. In spite of the large extent of published data dedicated to ethylene production by the Botrytis-host system and, in particular by *B. cinerea*, it is not very clear so far how and why the fungus produces ethylene.

Research performed in this field showed that *B. cinerea* is able to produce ethylene *in vitro*. Qadir *et al*. (1997) reported ethylene production from liquid cultures of *B. cinerea* grown in methionine-enriched media. However, using gas chromatography (GC) it is difficult to quantify the ethylene released by *B. cinerea* in the absence of methionine in the basal media and to offer a good description of ethylene emission over time. These data were revealed by using the more sensitive laser-based ethylene detector. We proved that *B. cinerea* produced low, but detectable levels of ethylene, when grown *in vitro* on PDA (potato dextrose agar) media without added methionine. A constant emission of 0.17 ± 0.04 nl h^{-1} was detected during the first 24 h for 160 μl of suspension at $2x10^7$ conidia ml^{-1} concentration. The ethylene production increased to a peak of about 1 ± 0.05 nl h^{-1} after 43 h from plating the conidia on PDA, after which it decreased to 0.2 ± 0.04 nl h^{-1}. As control we used 160 μl of autoclaved conidial suspension and autoclaved hyphae, respectively, plated on PDA (data not shown). In this case, no increase of ethylene emission was found over a period of 3 days. The equivalent ethylene production of the control, representing the background of non-enzymatically produced ethylene, showed a constant level of 0.18 ± 0.05 nl h^{-1} for the autoclaved conidial suspension, 0.18 ± 0.04 nl h^{-1} for autoclaved hyphae, and 0.17 ± 0.03 nl h^{-1} for PDA media alone, respectively. Addition of methionine greatly enhances the ethylene production by *B. cinerea* (Qadir *et al*., 1997; Cristescu *et al*., 2002; Chague *et al*., 2002). We inves-

tigated 15 concentrations of methionine and found that a small amount of L-methionine (0.05 mM) present in the PDA media already increased ethylene release by 3-fold. The pattern of ethylene production, showing a peak after approximately 43 hr, was similar as the pattern observed for the fungus grown on PDA without methionine. The highest ethylene production occurred in the presence of 3 to 15 mM L-methionine (around 30 nl h^{-1}). At higher levels it decreases with increasing methionine concentration, probably due to its effect on fungal vitality. These results differ from those reported by Qadir *et al*. (1997) who examined the effect of adding 1, 5, 10, 35 and 50 mM L-methionine in PDA and found a maximum ethylene production by fungus grown on PDA supplemented with 35 mM L-methionine. This may be caused by the fact that large errors are introduced by the integration method over 7 days ethylene production (Qadir *et al*., 1997).

Ethylene emission by *B. cinerea* was found to be dependent on the concentration of conidia plated on the growing media. The ethylene release was higher and the peak in ethylene production occurred earlier with increasing conidia concentrations (Fig. 3). Radial growth of the fungus was slightly lower for media containing methionine than for the media without methionine, although no significant variations could be observed between media with different methionine concentrations.

Addition of other ethylene precursors than methionine, such as 2-oxoglutarate or glutamate including their co-factors (e.g. ferric ions, L-arginine) in the growing media (either in liquid culture or in PDA) did not stimulate the ethylene released by *B. cinerea* indicating methionine-dependent synthesis (Qadir *et al*., 1997; Cristescu *et al*., 2002; Chague *et al*., 2002).

Using a pharmacological approach, we showed that *B. cinerea* most likely produces ethylene from methionine via the KMBA pathway (Cristescu, *et al*., 2002). Inhibitors of the plant ethylene pathway, such as amino oxyacetic acid (AOA) and amino ethoxy vinylglycine (AVG), had no effect on the ethylene emission from the fungus. Furthermore, using 2,4-dinitrophenylhydrazine as reagent, Chague *et al*., (2002) tested the presence of KMBA in different growth media. They found that KMBA was present only in the media supplemented with methionine, being produced by the fungus and secreted into the media. Additionally, no ACC synthase or ACC oxidase homologs have been found in *B. cinerea* genome while *Penicillium citrinum* has a functional ACC synthase gene (Jia *et al*., 1999). These observations show that *B. cinerea* does not use the plant ethylene pathway for ethylene synthesis.

Light microscopic analysis showed that conidial germination occurs within the first 3 hours after harvesting and plating, well before ethylene release becomes substantial. In fact, no ethylene was monitored during conidia dormancy and germination. Ethylene emission increased slowly during hyphae elongation, then very rapidly and at high rates till it reached the maximum production when the fungal hyphae extensively grew and began to branch (Cristescu *et al*., 2002). Thus, ethylene released by *B. cinerea* is associated with hyphal growth rather than conidial germination. Once it reaches a maximum, ethylene emission by the fungus shows a decline associated with further growth of the fungus (Cristescu *et al*., 2002). These results were supported by Chague *et al*. (2002) who found that KMBA is produced by young hyphae and not by dormant and germinating conidia. Moreover, older mycelium produces less KMBA and subsequently, less ethylene.

KMBA conversion

Ethylene production by *B. cinerea* is partly dependent on light. We found that ethylene produced by the fungus grown on methionine-enriched media was 5-fold higher under low light intensity conditions (5 µmol photons $m^{-2}s^{-1}$) than in dark (Fig. 3 inset).

Using the photoacoustic ethylene detector we were able to monitor the dynamics of ethylene released by *B. cinerea* while interchanging the light and dark regimes (Fig. 3). The ethylene emission from *B. cinerea* changes in less than 2 minutes after switching from light to dark and/or from dark to light (Fig. 3 inset). When the light is switched off, ethylene emis-

sion firstly shows a fast exponential decline during the first 30 minutes followed by a slower linear decrease. After switching back to light, ethylene release increases exponentially to levels that occurred before the dark treatment.

These data indicate that KMBA conversion to ethylene is considerably lower in the dark than in light. Similar results were reported by Chague *et al*. (2002). These authors hypothesized that in the dark KMBA accumulates in the medium and, upon switching on the light, the accumulated KMBA is rapidly converted into ethylene. However, their data show that the light-induced ethylene release following 24 h dark incubation is over 20 times less than expected considering the huge amounts that should have accumulated assuming continuous KMBA synthesis. This implicates that KMBA synthesis is apparently also affected by light/dark.

To explain the observed dynamics in fungal ethylene production, we need to consider the mechanism for KMBA conversion to ethylene (Fig. 1). A major role in this process is played by the free radicals which are generated in both dark and light conditions. In the dark, the radicals are formed by respiratory processes (chemical). In the light, additional radicals will be generated due to photochemical processes. In *B. cinerea* other sources may be considered for the formation of free radicals such as pigments and/or the reaction of light directly with chemical compounds present in the fungus cells.

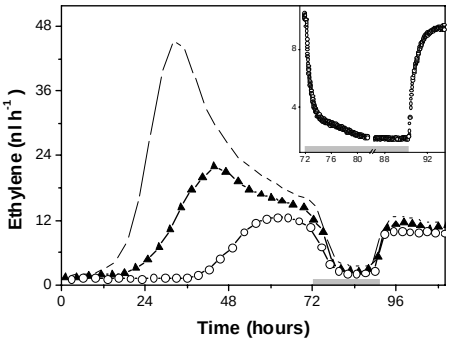

Figure 3. Ethylene released by *B. cinerea in vitro* at different concentrations $1.5x10^8$ (■), $2x10^7$ (▲) and $2x10^5$ (o) conidia ml^{-1} (160 µl) plated on PDA containing 25 mM L-methionine in light and dark (grey bar), respectively. Inset: the KMBA conversion to ethylene when switching from light to dark and back to light for $2x10^5$ (o) conidia ml^{-1} starts within 2 minutes.

Light seems to exert a dual action on ethylene release by *B. cinerea*. Firstly, light produces extra radicals which increase the conversion of KMBA to ethylene and secondly, the KMBA synthesis is mediated by action of a flavin, which may be stimulated by light.

The acceleration of ethylene release *in vitro* at higher conidia concentrations (see Fig. 3) also suggests that development of the fungus responds to chemical signals in its neighborhood. This could be related to sensing e.g. (self-produced) ethylene.

Since the ethylene precursor (methionine) is present in plant tissues, it may be used by the fungus as substrate for ethylene production via the KMBA pathway. The mechanism for ethylene production by *B. cinerea in planta* is rather complex due to the multiple ways to convert the fungus released KMBA. One such possibility may be furnished by the oxidative environment generated during the plant-fungus interaction. For example, the hydroxyl radicals that are produced together with other active oxygen species (AOS) can cause chemical oxidation of KMBA. It has been shown that *B. cinerea* produces hydrogen peroxide (H_2O_2) when grown on autoclaved flax stems (Bratt *et al*., 1988), possibly due to an oxidase activity, and H_2O_2 can be further converted to superoxide (O_2^-) and hydroxyl (OH•) radicals. In addition, Georgieva *et al*. (2000) reported an enhanced peroxidase activity in the tomato fruit pericarp upon infection. From here it raises naturally the question whether the *B. cinerea* ethylene significantly contributes to the total ethylene production in infected plant and if it plays a role in triggering the plant ethylene production (or manipulates ethylene production to trigger other defense mechanisms).

Ethylene perception by *B. cinerea*

It is likely that *B. cinerea*, like some other fungi, can sense ethylene and change its behavior accordingly. To gain more insight in the role of ethylene on fungal development, we moni-

tored ethylene released by *B. cinerea* grown on medium containing ethylene perception inhibitor silver thiosulphate (STS). We observed that in the presence of STS, the hyphal growth was reduced. Moreover, the increase in ethylene production was delayed and the maximum ethylene level was lower compared to *B. cinerea* growing on PDA without STS. This may indicate that ethylene perception mediates fungal growth (Fig.4).

Role of ethylene in plant-fungal interaction *in vivo*

Infection of tomato fruits with *B. cinerea* resulted in enhanced ethylene release which started to rise before visible decay development. This demonstrates that ethylene can be considered a sensitive marker for early infection in harvested fresh products (Cristescu *et al*., 2002; Polevaya *et al*., 2001). In a previous work it was shown that higher inoculum concentrations of *B. cinerea* increased infection when applied to flowers and to wounds caused by leaf removal (Eden *et al*., 1996). We found that also in the case of infected tomato fruit the decay development is dependent of the concentration of inoculum and it is faster for higher conidia concentrations (Cristescu *et al*., 2002). It is known that ethylene production induces fruit ripening and that ripe fruits and senescent or wounded plant organs are more susceptible to *B. cinerea*. Accordingly, we observed a faster disease development for the fast ripening tomato cultivar Money Maker accompanied by higher levels of ethylene

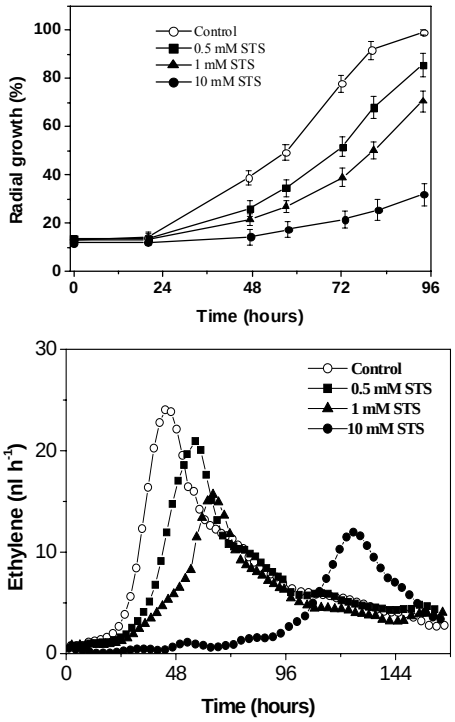

Figure 4. Ethylene production from *B. cinerea* (160 µl at $2x10^7$ conidia/ml) grown on PDA with 25 mM methionine without STS (Control) and with different STS concentrations, respectively. Up: the radial growth of the fungus grown in these conditions.

release compared to the slow ripening cultivar Daniela.

We found that exogenous application of ethylene (10, 20 ppbv and 1 ppmv of ethylene in air) did not affect the conidial germination or the hyphal growth of *B. cinerea in vitro*. In this case, the ethylene production by the fungus and its development were similar as in non-treated hyphae. Therefore, the presence of ethylene may represent an (indirect) advantage for the fungus, because it stimulates ripening and softening of the plant tissue and, therefore, facilitates tissue penetration and fungal spread (Diaz *et al*., 2002).

B. cinerea is able to engage various infection strategies depending on the infected host. For example, infection of tomato leaves with *B. cinerea* occurs in three phases (Benito *et al*., 1998): i). primary lesion formation characterized by a necrotic lesion appearance; ii). quies-

cent phase when no disease development or fungal growth can be seen and iii). lesion expansion phase when the fungus colonizes the whole host leaf. Our results indicate that infection in fruits can be described according to a similar scheme. As an example, we present the evolution of the ethylene emission from a tomato fruit, artificially inoculated with *B. cinerea* by four small infections (2 mm deep in the epidermis, 160 μl) at $2x10^7$ and $2x10^5$ conidia ml^{-1} (Fig.5).

The following pattern of ethylene production was repeatedly observed:

i). Initial inoculation (period 0-12 hours). A small peak in ethylene emission is observed both in *B. cinerea* and in mock infected tomato (not shown). However, ethylene production from *B. cinerea* infected tomato is more pronounced. Spore germination starts within the first 3 hours from inoculation. As no ethylene was produced by the fungus *in vitro* during its germination or dormancy, it is suggested that this first peak is part of the defense response by the attacked host (Ciardi *et al*., 2000).

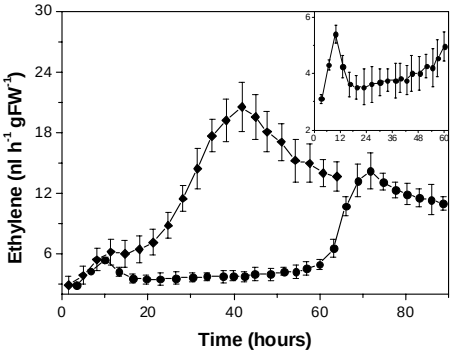

Figure 5. Ethylene production from tomatoes (FW = 80÷100g) infected with *B. cinerea* at $2x10^5$ (•) and $2x10^7$ conidia ml^{-1} (♦), respectively. At 0 h tomatoes were inoculated and immediately placed into cuvettes under continuous air flow of 4 liter h^{-1}. The inset shows the increase of ethylene emission from infected tomato with $2x10^5$ conidia ml^{-1} (■) for the first 2 days. Measurements were stopped when the fruits were completely deteriorated. Data are displayed as the averages of the sampling rate every 3 h (the errors due to averaging were smaller than the symbol size).

According to Robinson *et al*. (2001), this first peak is due to the conversion of the ACC existing in the tomato tissue, followed by increased transcription of the ACC synthase genes which will generate more ACC inside the fruit tissues, presumably used during the last stage of infection.

ii). Non symptoms stage (period 12-48 hours) when there are no visible disease symptoms. At the beginning of this period the fungus stays dormant; its growth is temporarily stopped due to the complex defense machinery activated by the tomato host. At a certain point the fungus switches to an invasive action. This corresponds to the moment of hyphae elongation which *in vitro* occurs after 18 hours. Soon the hyphae are branching (after 24 h) and ethylene starts to increase slowly.

iii). Visible disease development (after 48 h). A second peak in ethylene production, much larger than the first one, is recorded. This burst in ethylene release from infected tomato is a clear expression of disease development as a consequence of the tissue damage caused by *B. cinerea*. Senescence is initiated in this stage and the host lost the battle for its survival. The decrease in ethylene emission of the infected tomato after reaching the peak corresponds with an advanced stage of the fungal infection. It was suggested that during the infection process the infected tissue gradually loses the capacity to convert ACC to ethylene (Achilea *et al*., 1985).

Infection-related ethylene production by both tomato cultivars showed appropriate patterns as for the ethylene released by the fungus *in vitro*, although at much higher values (hundreds fold). Therefore, ethylene generation by infected tomatoes can be considered a likely response of the host to the stress caused by *B. cinerea* infection. Ethylene production by the fungus *in vitro* is very low even in comparison with ethylene released by mock-infected fruits to be considered substantial in inducing fruit spoilage. Moreover, enhanced formation of ethylene by the infected tomato was monitored well before ethylene released by the fungus *in vitro* started to increase. Our cytological analysis indicated that conidia germination and fungal growth inside the fruit were comparable

to those *in vitro.* These results indicate that the ethylene emission by the tomato-fungus system is not triggered directly by ethylene production of *B. cinerea*, although it is strongly "synchronised" with the growth rate of the fungus inside the tomato.

The first two stages of disease development, as described above, seem to be critical for the host because it has to generate adequate actions to successfully restrict or stop the fungus, such as production of phytoalexins and other phenolic compounds, pathogenesis-related proteins including those against the cell wall degrading enzymes (CWDEs) (Benito *et al*., 1998), polygalacturonase inhibiting proteins (Johnson, 1994), AOS (Baker, 1995; Levine, 1994). As for the fungus, it has to cope with the oxidative stress induced by the host and counteract the host produced compounds which inhibit its growth (Pezet at al., 1991). A complex and dynamic pattern of H_2O_2 formation was observed within tomato and bean leaves early after inoculation with *B. cinerea*. In the early stage of the infection the H_2O_2 originates from the host cells as an induced defense reaction, while after penetration of the epidermal cell wall, additional H_2O_2 may be generated directly by the fungal enzymes. A considerable increase in cytosolic H_2O_2 was found between 5-24 h after inoculation of tomato leaves with *B. cinerea* and apoplastic generation, as indicated by NADH peroxidase activity, was enhanced between 24 and 72 h (Patykowsky and Urbanek, 2003). It was suggested that *B. cinerea* experiences H_2O_2 stress only in the early stage of infection. At this time, the fungus had penetrated the host, but the infection remained symptomless (Schouten *et al*., 2002a). As a consequence of the oxidative stress imposed, *B. cinerea* temporarily exhibits a decrease in growth.. Recently, Malolepsza and Urbanek (2000) showed that mycelial growth of *B. cinerea* was completely inhibited at 100 mM H_2O_2 (not checked if the fungus was killed or just temporarily impaired in growth), while others reported that germination of conidia occurred in the presence of 180 mM H_2O_2 and both germination and fungal development was slowed down, but not inhibited by up to 1 M H_2O_2. Moreover, *B. cinerea* is able to produce both intra- and extracellular enzymes (i.e. superoxide dismutase, laccase, catalase, different peroxidase to inactivate the H_2O_2 (Gil-ad *et al*., 2000). The intracellular enzymes may only serve to protect the fungus from its own AOS, while the extracellular ones may be involved in protecting the fungus against the AOS from the host plant. It was recently postulated that *B. cinerea* actively triggers the production of AOS in planta in order to kill host cells, thereby facilitating entry into host. In leaves of *A. thaliana* infected by *B. cinerea*, massive depletion of ascorbic acid levels occurred before visible infection as a result of damage to the antioxidant mechanism (i.e. redox status) represents an early event in the infection process (Muckenschnabel *et al*., 2002). In the mitochondrial fraction a continuous decrease in activity of ascorbate peroxidase (APX) -one of the major H_2O_2-decomposing enzymes in plant cells was observed in the inoculated leaves (Kuzniak and Sklodowska, 2004). They found that GSH (glutathione) and AA (ascorbate) pools together with the ascorbate-related enzymatic reactions were heavily suppressed once the spreading lesions started to develop.

The three stages described above were observed when low concentrations of the inoculation were used (in the order of 10^5 conidia/ml or lower). For higher concentrations the second stage of non symptoms is very short and from the ethylene perspective, its production presents a continuous and rapid rise from beginning toward the third stage.

The low ethylene production of the fungus compared to the production of the tomato-fungus system already indicated that the contribution of fungal ethylene to the total ethylene is negligible. However, while invading the host, the fungus may have access to increased amounts of methionine or alternative substrates that increase its ethylene production. Therefore, tomato slices were treated with inhibitors of plant (not fungal) ethylene production and thereafter infected with *B. cinerea*. Inhibition of ethylene biosynthesis in *Botrytis*-infected tomato slices with AOA applied prior to inoculation significantly decreased the ethylene emission. However, it did not block it completely (Cristescu *et al*., 2002). This remain-

ing activity may be due to either plant ethylene in the case the inhibitor is not 100% effective or, alternatively, it may be due to fungal ethylene production. To determine the efficiency of the inhibitor treatment, the experiments were repeated with AVG, while also a wound control was inserted to determine the efficiency of the applied AVG (Fig. 6). AVG-treated slices were severely wounded and their ethylene production compared to wounded non-pretreated slices. Wounded slices produced a significant amount of ethylene that was almost completely blocked by prior AVG treatment. Ethylene production in AVG treated and then *B. cinerea* infected slices was blocked to the same extent, which clearly shows that virtually all the ethylene produced in the plant-pathogen system is derived through ACC and, therefore of plant origin. The fungal growth was slightly reduced in the AVG treated tissues, although it recovered rapidly.

A control experiment where the fungus was allowed to grow on a medium containing autoclaved grounded tomato tissue showed no increased ethylene production by the fungus (unpubl.). To the contrary, ethylene production was consistently lower on tomato medium than on PDA medium. This indicates that tomato tissue is not a good substrate for ethylene production by *B. cinerea*. The presumed chemical conversion of KMBA to ethylene may be suppressed by the presence of e.g. antioxidants in the tomato medium

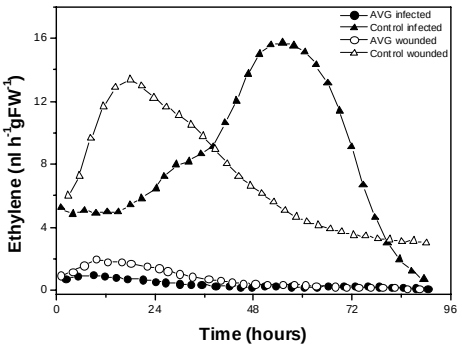

Figure 6. Ethylene production from slices of tomatoes treated with AVG (0.5 mM) compared to the non-treated fruits. Following 3 h AVG treatment, slices were either wounded or inoculated with 10^5 conidia/ml.

The question which arises is whether ethylene production in *B. cinerea* infected tomato is an autocatalytic process and whether traces of ethylene produced through fungal KMBA may trigger plant ethylene production. Experiments were performed with MCP pre-treated tomatoes that were later inoculated with *B. cinerea*.

The experiments revealed that ethylene levels monitored in this case are comparable to or slightly higher than those from non-treated infected tomatoes. In MCP treated tomato, fungal ethylene (if produced at all) will not be able to trigger the plant ethylene production. If the (initially) produced ethylene would be important for further ethylene production, the MCP tomato should show much less ethylene especially at later stages of infection. Because this was not the case, we indicate that ethylene produced by tomato in response to *B. cinerea* infection is rather directly elicited by other mechanisms initiated by the fungus than by autocatalysis. One such mechanism may be the production by the fungus of necrosis- and ethylene-inducing protein (NEP) that directly induces ethylene production in the attacked host. The NEPs and their homologues have been reported in many fungal species, like *Verticillium* (Wang *et al*., 2004), *Fusarium* (Bailey *et al*., 1994), oomycete species, including *Phytophthora* and some eubacteria (Qutob *et al*., 2002; Fellbrich *et al*., 2002). Whether this is the case in *B. cinerea* infection remains to be elucidated. In this context, ethylene apparently plays a role in plant resistance. Interestingly, we observed a significant increase of disease development in the MCP-treated tomatoes compared to the non-treated fruits as it was also reported by Diaz *et al*. for disease development in leaves (2002).

ETHYLENE AND RESVERATROL

One of the challenges to the modern agriculture is to deal with the enormous post-harvest losses of fresh products which may add up to 30% (Kader *et al*. 1992). These losses are mainly due to product deterioration as a result of over-ripening, senescence or pathogen attack. The use of low temperature storage, controlled

atmosphere (CA) conditions and various pesticides is a common solution to overcome these problems, however, not without human health risks and environmental consequences. New strategies based on exploitation of the natural plant capabilities to improve its defense mechanisms and, hence, resistance may decrease the post-harvest losses as a result of pathogen attack. In this context, resveratrol (3, 5, 4′-trihydroxystilbene) takes an important place because of its implications in both phytopathology and human health. This compound is naturally produced as phytoalexin in grapevine, peanut and other plants and it was found to protect the host against fungal infections (Breuil *et al*., 1999). These include fungi as *Plasmopara viticola* (Dai. *et al*., 1995), *Phomopsis viticola* (Hoos and Blaich, 1990) or *Rhizopus stolonifer* (Sarig *et al*., 1997). Because the compound is effective against a broad range of fungal species and the selective accumulation of resveratrol in grape skin make it a good candidate as a natural pesticide against pathogen attack as a result of improvement of the natural resistance of grapes.. Due to its antioxidant properties, resveratrol can also have positive effects on fruit conservation during storage as it may slow down the deteriorative processes. Consequently, both endogenous enhancement and exogenous application could be exploited to reduce grape spoilage.

Since it was reported that *B. cinerea* can elicit the production of resveratrol in grapevines (Langcake and Pryce, 1976), many investigations have been carried out on this particular host-pathogen interaction (Stein and Blaich, 1985; Jeandet *et al*, 1995, Adrian *et al*., 2000, Breuil *et al*., 1998; Montero *et al*., 2003). In the wine industry, the growth of *B. cinerea* on wine grapes has been known as "noble rot" which gives an added effect to the bouquet of certain wines. Nevertheless, the grey mold is more often a severe problem for all grape varieties; the fungus can settle in on immature grapes and during the humid periods early in the season continues to penetrate the grapes causing them to rot.

Resistance of grapevines to *B. cinerea* infection is the result of multiple defense mechanisms consisting mainly of accumulation of phytoalexins, such as resveratrol, and the synthesis of pathogenesis related (PR)-proteins (Derckel *et al*., 1999). Adrian *et al*. (1998) showed that in the presence of resveratrol conidia germination as well as mycelium growth of *B. cinerea* were significantly reduced. In response to the enhanced levels of resveratrol, the fungus produces blue-copper oxidases known as stilbene oxidases or laccases which were believed to detoxify resveratrol. By means of functional molecular genetic analysis of *B. cinerea* laccases, Schouten *et al*. (2002b) found a resveratrol-induced laccase gene *Bclcc2* which paradoxically is responsible for transforming resveratrol into fungitoxic compounds, thus, producing self-intoxication. In spite of this obvious advantage for the host, in the long term, it seams that the fungus can profit from the expression of this gene, because there are no *B. cinerea* strains reported so far in which this gene is deleted or its expression repressed. Thus, both the fungus and the plant-host can mutually benefit from this mechanism.

Ethylene is involved in the ripening process of many fruits and it may also play a role in pathogenesis. During the ripening phase of climacteric fruits (e.g. apples, tomatoes, etc.) both CO_2 and ethylene are emitted at elevated levels as opposed to non-climacteric fruits (e.g. citrus). In grapes it was reported that the resveratrol content decreases during ripening and, therefore, the fruit become more susceptible to *B. cinerea* infection (Sarig *et al*., 1997). As non-climacteric fruit, grapes release ethylene at very low production rate (Archbold *et al*., 1997); almost undetectable with standard procedures. The use of the laser-based ethylene detector however, enabled us to investigate the dynamics of ethylene evolution in grapes with much improved accuracy. This device was simultaneously complemented by another laser-based instrumentation that uses Laser Desorption (LD) coupled with Laser Resonant Multiphoton Ionisation with Time-of-Flight Mass Spectrometric detection (REMPI-TOFMS) that provided fast and direct analysis of resveratrol in grapes (Montero *et al*. 2000, 2000b, Orea *et al*., 2001). As resulting from this combination, we provided the first report on the real-time monitoring of ethylene by *B. cinerea* in-

fected grapes in association with resveratrol levels (Montero *et al*., 2003) (Fig. 7).

In the case of non-infected grapes, the resveratrol content and the ethylene released showed an opposite behaviour with respect to each other; high resveratrol content corresponds to a low ethylene emission. Interestingly, the resveratrol content from the non-infected fruits was higher than that corresponding to the mock-infected fruits, which drastically decreased to zero during the first day after the buffer inoculation. In correlation, ethylene released by mock-infected grapes increased in the first day up to a certain level and showed higher values compared to the non-infected fruits. For the *Botrytis*-infected fruits, the resveratrol content increased to a maximum on the second day after infection, followed by a fast decrease, most probably due to its oxidation by the *B. cinerea* laccase. At the time the resveratrol content showed a decline, the ethylene emission started to rise. Overall, it seems that in grapes, the resveratrol content is determining the rate of ethylene production.

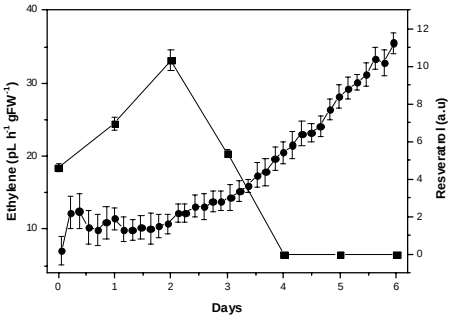

Figure 7. Ethylene production by grapes infected with *B. cinerea* (5 µl of the suspension at 10^3 conidia mL^{-1} per grape inoculated at 0 h) (•) and evolution of *trans*-resveratrol content (■) in grape skin of *Botrytis* infected grapes.

The relation is not reciprocal, because as it was described previously, continuous exposure to exogenous ethylene did not affect gray mold nesting ability on table grapes artificially inoculated with *B. cinerea* (Palou *et al*., 2002, 2003). Previous investigations on the production of resveratrol by grapes in response to *Botrytis* infection (Jeandet *et al*., 1995, Adrian *et al*., 2000) showed that its elicitation occurred predominantly in the non-infected grapes surrounding the infected ones, while in the latter the resveratrol content was always lower than in the non-infected grapes. This apparently contradictory result is due to the time of resveratrol analysis which was done several days after the *Botrytis* infection when, as the authors claim, resveratrol could have been already metabolized by the fungus (as it happened in the present case after the second day). In accordance to our study, Paul *et al*. (1998) reported the induction of resveratrol by *B. cinerea* in leaves which reached a maximum yield in the third day after infection, followed by a rapid reduction of the resveratrol content by the fifth day.

The activity of resveratrol as natural pesticide has been investigated by exogenous application on grapes. A short submerge (5 s) of the grapes in 1.6 x 10^{-4} M solution of resveratrol affected the ethylene production in two directions: (1) delaying the enhanced ethylene emission with about 2 days and (2) decreasing the ethylene production of at least 3-fold. This significant decrease of the ethylene production in the treated grapes can be attributed to the action of trans-resveratrol on different microorganisms (bacteria and fungi) present on the grapes. This hypothesis is supported by a recent work on the effects of *B. cinerea* on grapes (Dorado *et al*., 2001), performed in similar experimental conditions and with the same variety of grapes as in the present study. According to this report, other microorganisms like bacteria and fungi, distinct from the inoculated *Botrytis*, were developed during the incubation period of grapes and caused the deterioration of the fruits. The identified non-inoculated microorganisms present on grapes were mainly yeasts and imperfect fungi such as *Penicillium*, *Aspergillus* and *Alternaria spp.* which are known to include ethylene-producing species (Fukuda *et al*., 1993). This treatment has positive effects on fruit conservation during storage; it doubled the normal shelf-life of grapes at room temperature, maintaining their post-harvest quality for 10 days. This result offers a new, simple and inexpensive modality which can be used to improve the shelf-life of fruits and to preserve their

natural post-harvest quality. Recently, it was reported that exogenous application of resveratrol reduced post-harvest decay also in other types of fruits than grapes, such as tomatoes, apples, avocado, pears and papers (Jimenez *et al*., 2005).

Preventing water losses is a very important issue during the post-harvest period of fresh plant products. In this respect, it is suggested that resveratrol acts like a thin coat on the fruit, which not only protects against the microbial growth, but it also reduces water losses, thus conserving the water content and fruit firmness. Additionally, it was demonstrated that application of resveratrol to several fruit does not alter their organoleptic and biochemical properties (González Ureña *et al*., 2003).

Microbial contamination of food is one of major problems with risks for the human health. Although some reports intended to show that risks related to using natural chemicals in foods are even greater than the risks from pesticide residues (Pimentel *et al*., 1996, Swirsky *et al*., 1997), the lack of toxicity of the resveratrol has been demonstrated. A considerable number of investigations are currently focussed on the health benefits of resveratrol consumption (see Frémont 2000, German and Walzem, 2000 or Parr and Bowell, 2000 for recent reviews on this subject) giving it an additional value as candidate for bio-control experiments against *B. cinerea*, as better alternative than the use of harmful chemical pesticides.

CONCLUSIONS

We have shown that a laser-based ethylene detector represents a suitable instrumentation for on-line measurements of ethylene released by fungi *in vitro* and *in vivo*. Moreover, the instrument is a powerful tool for the early detection of traces of ethylene released in the case of infection caused by microorganisms (fungi or bacteria) with long period of incubation (weeks).

Its high sensitivity and fast time response allow to investigate the temporal and functional relationship between fungal and plant ethylene biosynthesis. In addition, this method eliminates the large data variability which might be generated during the use of the standard instrumentation (e.g. GC-gas chromatography) due to the integration procedure over many hours/days.

This development may lead to future applications in the post-harvest technologies based on alternative strategies for fresh produce protection, mainly focused on action on the fungus rather that on inhibition plant-produced ethylene, that usually associate enhanced shelf life with decreased flavor and quality. Among ethylene, other components of biological interest can be monitored in real-time with the laser-based photoacoustic detectors. A trend in fruit storage is to reduce the oxygen level in order to slow down ripening and senescence. In this way, the aerobic respiration is gradually replaced by alcoholic fermentation that leads to production of acetaldehyde and ethanol. Alcoholic fermentation is also connected with stress-signal transduction and the disease-resistance response in plants. Several plant species when exposed to environmental stress such as water deficit, low temperature, ozone exposure or pathogen infection can generate significant amounts of acetaldehyde and ethanol at ambient of even at elevated oxygen concentrations (Tadege and Kuhlemeier, 1997). Laser-based photoacoustic detectors have been proven to be able of sensitive detection of acetaldehyde and ethanol at and below the part per billion level (Zuckermann *et al*., 1997b). These compounds were monitored as markers for alcoholic fermentation in (pos)anoxic fruits (Zuckermann et.al., 1997b; Oomens *et al*., 1998), (post)submerged rice seedlings (Boamfa *et al*., 2003), wheat dough (Tomas *et al*., 2001), dehydrated radicles of cucumber and pea (Leprince *et al*., 2000) and poplar trees (Kreuzwieser *et al*., 2001).

In many cases, due to a disturbed balance between the formation of AOS in plant tissue as a result of stress and the normal scavenging capacity, the plant tissues suffer from e.g. lipid peroxidation that can cause damage of cell membranes (Halliwel and Gutteridge, 1989). The gaseous end products of this process are ethylene and ethane which can be sensitively

monitored by laser-based photoacoustics. Ethane was monitored as a result of photo-oxidative damage of chilled cucumber leaves (Santosa *et al*., 2003), membrane peroxidation in pears (Veltman, *et al*., 1999), lipid peroxidation induced during artificial aging of onion seeds (Klein *et al*., 2004).

As mentioned previously, the plant recognition of pathogen infection leads to so called hypersensitive response (HR) indicated by a fast, localized cell death at the site of infection. A synergisticy mechanism has been proposed between the AOS and nitric oxide (NO) during the HR response in plants and using a CO laser-based photoacoustic detector, the first in planta and direct measurements of NO emission from plants undergoing various responses to *P. syringae* challenge has been obtained (Mur *et al*., 2005).

These examples clearly show the high potential of photoacoustic detection systems for studying plant metabolism and emphasize the many possibilities for detailed studies of plant-pathogen interactions.

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REFERENCES

Abeles, F. B. (1973). Ethylene in plant Biology. Academic Press. New York, p. 302.

Abeles, F. B., Morgan, P. W., and Saltveit, Jr, M. R. (1992). Ethylene in plant biology, 2nd ed. Academic Press, San Diego, CA.

Achilea, O., Fuchs, Y., Chalutz, E., and Rot, I. (1985). The contribution of host and pathogen to ethylene biosynthesis in *Penicillium digitatum*. Physiological Plant Pathology 27:55-63.

Adrian, M., Rajaei, H., Jeandet, P., Veneau, J., and Bessis, R. (1998). Resveratrol oxidation in *Botrytis cinerea* conidia. Phytopathology 88:472-476.

Adrian, M., Jeandet, P., Douillet-Breuil, A. C., Tesson, L., and Bessis, R. (2000). Stilbene content of mature *Vitis vinifera* berries in response to UV-C elicitation. J.ournal of Agricultural and Food Chemistry 48:6103-6105.

Amagai, A., and Maeda, Y. (1992). The ethylene action in the development of cellular slime molds: an analogy to higher plants. Protoplasma 167:159-168.

Archbold, D. D., Hamilton-Kemp, T. R., Barth, M. M., and Langlois, B. E. (1997). Identificating natural volatile compounds that control gray mold (*Bot. Cin*.) during post-harvest storage of strawberry, blackberry and grape. Journal of Agricultural and Food Chemistry 45:4032-4037.

Bailey, J., Jennings, C., and Anderson, J. D. (1997). The 24-KDa protein from *Fusarium oxysporum f. sp. erythroxyli*: occurrence in related fungi and the effect of growth medium on its production. Canadian Journal of Microbiology 43:45–55.

Baker, C. J., and Orlandi, E. W. (1995).Active oxygen in plant pathogenesis. Annual Review of Phytopathology 33:299-321.

Bashan, Y. (1994). Symptom expression and ethylene production in leaf blight of cotton caused by *alternaria macrospora* and *Alternaria alternate* alone and combined. Canadian Journal of Botany 72:1574-1579.

Barkai-Golan, R., Lavy-Meir, G., and Kopeliovitch, E. (1988). Pectolytic and cellulolytic activity of *Botrytis cinerea. Pers*. related to infection of non-ripening tomato mutants. Journal of Phytopathology 123:174-183.

Bell, A. A. (1981). Biochemical mechanisms of disease resistance. Annual Reviews of Plant Physiology 32:21-81.

Benito, E. P., ten Have, A., van 't Klooster, J. W., and van Kan, J. A. L. (1998). Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. European Journal of Plant Pathology 104:207-220.

Bent, A., Innes, R., Ecker, J., and Staskawicz, B (1992). Disease development in ethylene –insensitive Arabidopsis thaliana infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. Molecular Plant-Microbe Interactions 5:372-378.

Biles, C. L, Abeles, F. B., and Wilson, C. L. (1990). The role of ethylene in anthracnose of cucumber, *Sucumis sativus,* caused by *Colletotrichum lagenarium*. Phytopatology 80:732-736.

Bijnen, F. G. C., Reuss, J., and Harren, F. J. M. (1996). Geometrical optimization of a longitudinal resonant photoacoustic cell for sensitive and fast trace gas detection. Rev. Sci. Instr. 67:2914-2923.

Boamfa, E. I., Ram, P. C., Jackson, M. B., Reuss, J., and Harren, F. J. M. (2003). Dynamic aspects of alcoholic fermentation of rice seedlings in response to anaerobiosis and to complete submergence: relationship to submergence tolerance. Annals of Botany 91:279-290.

Boller, T. (1988). Ethylene and the regulation of antifungal hydrolases in plants. Oxford Surveys on Plant Molecular and Cell Biology 5:145-174.

Boller, T. (1991). Ethylene in pathogenesis and disease resistance, p. 293-324. *In* A. K. Mattoo and J. C. Suttle (ed.), The plant hormone ethylene. CRC Press, Boca Raton, Fla.

Bratt, R. P., Brown, A. E., and Mercer, P. C. (1988). A role for hydrogen peroxide in degradation of flax fibre by *Botrytis cinerea.* Transactions of the British Mycological Society 91:481-488.

Breuil, A. C., Adrian, M., Pirio, N., Meunier, P., Bessis, R., and Jeandet, P. (1998). Metabolism of stilbene phytoalexins by *Botrytis cinerea*: 1. Characterization of a resveratrol dehydromer.Tetrahedron Letters 39:537-540.

Breuil, A. C., Jeandet, P., Adrian, M., Chopin, F., Pirio, N., Meunier, P., and Bessis, R. (1999). Characterization of a pterostilbene dehydrodimer produced by laccase of *Botrytis cinerea*. Phytopathology 89:298-302.

Brown, G. E., and Burns, J. K. (1998). Enhanced activity of abscission enzymes predisposes oranges to invasion by *Diplodia natalensis* during ethyelene degreening. Postharvest Biology and Technology 14:217-227.

Brown, G. E., and Lee, H. S. (1993). Interaction of ethylene with citrus stem-end rot caused by *Diplodia natalensis.* Phytopathology 83:1204-1208.

Brewer, R. J., Bruce, C. W., and Mater, J. L. (1982). Optoacoustic spectroscopy of C_2H_4. Applied Optics 21:4092-4100.

Catlett, N. L., Yoder, O. C., and Turgeon, B. G. (2003). Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Eukaryotic Cell 2:1151-1161.

Chague, V., Elad, Y., Barakat, R., Tudzynski, P., and Sharon, A. (2002). Ethylene biosynthesis in *Botrytis cinerea*. FEMS Microbiology Ecology 40:143-149.

Chang, C., and Stadler, R. (2001). Ethylene hormone receptor action in *Arabidopsis*. BioEssays 23:619-627.

Chappell, J. Hahlbrock, K., and Boller, T. (1984). Rapid induction of ethylene biosynthesis in cultured parsley cells by fungal elicitor and its relationship to the induction of phenylalanine ammonia-lyase (*Phytophthora megasperma).* Planta 161:475-480.

Chen, N., Goodwin, P. H., and Hsiang, T. (2003). The role of ethylene during the infection of *Nicotiana tabacum* by *Colltotrichum destructivum.* Journal of Experimental Botany 54:2449-2456.

Ciardi, J. A., Tieman, D. M., Lund, S. T., Jones, J. B., Stall, R. E., and Klee, H. L. (2000). Response to *Xanthomonas campestris* pv. *Vesicatoria* in tomato involves regulation of ethylene receptor gene expression. Plant Physiology 123:81-92.

Cohn, J., Sessa, G., and Martin, G. B. (2001). Innate immunity in plants. Current Opinion in Immunology 13:55-62.

Cristescu, S. M., de Martinis, D., te Lintel Hekkert, S., Parker, D. H., and Harren, F. J. M. (2002). Ethylene production by *Botrytis cinerea in vitro* and in tomatoes. Applied and Environmental Microbiology 68:5342-5350.

Cronshaw, D. K., and Pegg, G. F. (1976). Ethylene as a toxin synergist in *Verticillium* wilt of tomato. Physiological Plant Pathology 9:33-38.

Dai, G.H., Andary, C., Mondolot-Cosson, L. and Boubals, D. (1995). Histochemical Studies on the Interaction between three species of grapevine, Vitis Vinifera, V. rupestris and V. rotundifolia and the downy mildew fungus, Plasmopara viticola. Phys. and Molec. Plant Pathol,. 46:177-188.

Daundasekera, M. Joyce, D. C., Aked, J., Adikaram, N. K. B. (2003). Ethylene production by *Colletotrichum musae in vitro*. Physiological and Molecular Plant Pathology 62:21-28.

de Vries, H. S. M., Harren, F. J. M., and Reuss, J. (1995). In situ, real-time monitoring of wound-induced ethylene in cherry tomatoes by two infrared laser-driven systems. Post-Harvest Biology and Technology 6:275-285.

de Vries, H. S. M., Wasono, M. A. J., Harren, F. J. M., Woltering, E. J., van der Valk, H. C. P. M., and Reuss, J. (1996). Ethylene and CO2 emission rates and pathways in harvested fruits investigated, in situ, by laser photodeflection and photoacoustic techniques. Post-Harvest Biology and Technology 8:1-10.

Derckel, J. P., Baillieul, F., Manteau, S., Audran, J. C., Haye, B., Lambert, B., and Legendre, L. (1999). Differential Induction of Grapevine Defenses by two strains of *Botrytis cinerea*. Biochemistry and Cell biology 89:197-203.

Diaz, J., ten Have, A., and van Kan, J. A. L. (2002). The role of ethylene and wounding signalling in resistance of tomato to *Botrytis cinerea.* Plant Physiology 129:1341-1351.

Dixon, R. A., and Lamb, C. J. (1990). Molecular communication in interactions between plants and microbial pathogens. Annual Review of Plant Physiology and Plant Molecular Biology 41:339-367.

Dorado, M., Bermejo, E. González, J. L., Sánchez, A., and Luna, N. (2001). Development influence of *Botrytis cinerea* on grapes. Advances in Food Chemistry 23:153-159.

Eden, M. A., Hill, R. A., Beresford, R., and Stewart, A. (1996). The influence of inoculum concentration, relative humidity, and temperature of infec-

tion of greenhouse tomatoes by *Botrytis cinerea*. Plant Pathology 45:795-806.

El-Kazzaz, M. K., Sommer, N. F., and Kader, A. A. (1983). Ethylene effects on *in vitro* and *in vivo* growth of certain postharvest fruit-infecting fungi. Phytopatology 73:998-1001.

Elad, Y. (1990). Production of ethylene by tissue of tomato, pepper, French-bean and cucumber in response to infection by *Botrytis cinerea.* Physiollogical Molecular Plant Pathology 36:277-287.

Elad, Y. (1993). Regulators of ethylene biosynthesis or activity as a tool for reducing susceptibility of host plant tissues to infection by *Botrytis cinerea*. Netherlands Journal of Plant Pathology 99:105-113.

Elad, Y. (2002). Ethylene and reactive oxygen species in a plant-pathgen system. Phytoparasitica 30:307

Elad Y. and Eversen, K. (1995). Physiological aspects of resistance to *Botrytis cinerea*. Phytopathology 85:637-643.

Fan, X. T., Mattheis J. P., and Roberts, R. G. (2000). Biosynthesis of phytoalexin in carrot root requires ethylene action. Physiologica Plantarum 110:450-454.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Englehardt, S., Felix, G., Kemmerling, B. Krzymowska, M., and Nurnberger, T. (2002). NPP1, a *Phytophthora*-associated trigger of plant defense in *Arabidopsis*. Plant Journal 32:375–390.

Frémont, L. (2000). Biological effects of resveratrol. Life Sciences 66:663-673.

Fukuda, H., Kitajima, H., Fujii, T., Tazaki, M., and Ogawa, T. (1989). Purification and some properties of novel ethylene-forming enzyme produced by *Penicillium digitatum*. FEMS Microbiology Letters 59:1–6.

Fukuda, H., Takahashi, M., Fujii, T., Tazaki, M., and Ogawa, T. (1989). An NADH: Fe(III) EDTA oxidoreductase from *Cryptococcus albidus*: an enzyme involved in ethylene production *in vivo*? FEMS Microbiology Letters 60:107–112.

Fukuda, H., Ogawa, T., and Tanase, S. (1993). Ethylene production by microorganisms, p. 275–306. *In* A. H. Rose (ed.), Advances in microbial physiology, vol. 35. Academic Press Inc., London.

Georgieva, I., Edreva, A., Rodeva, R., Sotirova, V., and Stoimenova, E. (2000). Metabolic changes in tomato fruits and seeds after viral, bacterial and fungal infection. Acta Physiologica Plantarum 22:281-284.

Geraats, B. P. J., Bakker, P. A. H. M., and van Loon, L. C. (2002). Ethylene insensitive impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*. Molecular Plant-Microbe Interactions 15:1078-1085.

German, J. B., and Walzem, R.L. (2000). The health benefits of wine. Annual Review of Nutrition 20:561-593.

Gil-ad, N. L., Bar-Nun, N., Noy, T., Mayer, A. M. (2000). Enzymes of *Botrytis cinerea* capable of breaking down hydrogen peroxide. FEMS Microbiology Letters 190:121-126.

Gonzales Ureña, A., Orea, J. M., Montero, C., Jimenez, J. B., Gonzalez, J. L., Sanchez, A., and Dorado, M. (2003). Improving the post-harvest resistance in fruits by external application of *trans*-resveratrol. Journal of Agricultural Food Chemistry 51:82-89.

Govrin, E. M., and Levine, A. (2000). The hypersensitive reaction facilitates plant infection by the necrothophic fungus *Botrytis cinerea.* Current Biology 10:751-757.

Halliwell, B., and Gutteridge, J. M. C. (1989). Free radicals in Biology and medicine. Oxford: Clarendon press: 188-276.

Harren, F. J. M., and Reuss, J. (1997). Photoacoustic spectroscopy, p. 413-435. *In* G. L. Trigg (ed.), Encyclopedia of applied physics, vol. 19. VCH Publishers, Inc., Weinheim, Germany.

Hoffman, T., Schmidt, J. S., Zheng, X., and Bent, A. F. (1999). Isolation of ethylene-insensitive soybean mutants taht are altered in pathogen susceptibility and gene-for-gene disease resistance. Plant Physiology 119:935-950.

Hoos,G., and Blaich, R. (1990). Influence of resveratrol on germination of conidia and mycelial growth of *Botrytis cinerea* and *Phomopsis viticola*. J ournal of Phytopathology 129:102-110.

Hyodo, H. (1991). Stress/wound ethylene. In: Mattoo, A. K., and J. C. Suttle, Editors. The plant hormone ethylene. CRC Press, p. 65-80.

Ince, J. E., and Knowles, C. J. (1986). Ethylene formation by cell-free extracts of *Escherichia coli*. Archives of Microbiology 146:151-158.

Jarvis, W. R. (1977). *Botryotinia* and *Botrytis* species: taxonomy and pathogenicity. Monogr. Res. Branch Can. Dept. Agric. 15, Harrow, Ontario, Canada.

Jeandet, P., Bessis, R., Sbaghi, M., and Meunier, P. (1995). Production of the phytoalexin Resveratrol by grapes as a response to *Botrytis* attack under natural conditions. Journal of Phytopathology 143:135-139.

Jia, Y. (1999). Synthesis and degradation of 1-aminocyclopropane-1-carboxylic acid by *Penicillium citrinum*. Bioscience Biotechnology and Biochemistry 63:384-387.

Jimenez, J. B., Orea, J. M., Montero, C., Gonzales Ureña, A., Navas, E., Slowing, K., Goez-Serranillos, M. P., Carretero, E., and de Martinis, D. (2005). Resveratrol treatment controls micro-

bial flora, prolongs shelf life and preserves nutritional quality of fruit. Journal of Agricultural and Food Chemistry 53:1526-1530.

Johnson, P., and Ecker, J. (1998). The ethylene gas signal transduction pathway: A molecular perspective. Annual Review of Genetics 32:227-254.

Kader, A. A. (1985). Ethylene-induced senescence and physiological disorders in harvested horticultural crops. HortScience 20:54-57.

Kader, A. A. (1992). Postharvest biology and technology: an overview. In: Kader, A.A. Postharvest technology of horticultural crops. 42, 2nd ed. University of California, Oakland, CA, p. 15–20.

Kende, H. (1993). Ethylene biosynthesis. Annual Review of Plant Physiology 44:283-307.

Kepczynska, E. (1989). Ethylene requirement during germination of *Botrytis cinerea* spores. Physiology Plantarum 77:369-372.

Kepczynska, E. (1993). Involvement of ethylene in the regulation of growth and development of the fungus *Botrytis cinerea Pers. ex. Fr.* Plant Growth Regulators 13:65-69.

Kepczynski, J., and Kepczynska, E. (1977). Effect of ethylene on germination of fungal spores causing fruit rot. Fruit Science Reports 4:31-35.

Klein, J. D., Santosa, E., Laarhoven, L. J., Boamfa, E. I., Hebbe, Y., and Harren, F. J. M. (2004). Acta Horticulturae 631:39-42.

Knoester, M., van Loon, L. C., van den Heuvel, J., Henning, J., Bol, J. F., and Linthorst, H. J. M. (1998). Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proceeding of the National Academy of Sciences USA 95:1933-1937.

Kolattukudy, P. E., Rogers, L. M., Li, D., Hwang, C. S., and Flaishman, M. A. (1995). Surface signaling in pathogenesis. Proceedings of the National Academy of Sciences of the USA 92:4080-4087.

Kreuzwieser, J., Harren, F. J. M., Laarhoven, L. J. J., Boamfa, I., te Lintel-Hekkert, S., Scheerer, U., Hüglin, C., and Rennenberg, H. (2001). Acetaldehyde emission by the leaves of trees - correlation with physiological and environmental parameters. Physiologia Plantarum 113:41-49.

Kuzniak, E., and Sklodowska, M. (2004). The effect of *Botrytis cinerea* infection on the antioxidant profle of mitochondria from tomato leaves. Journal of Experimental Botany 55:605-612.

Langcake, P., and Pryce, R. J. (1976). The production of resveratrol by *Vitis vinifera* and other members of the *Vitaceae* as a response to infection or injury. Physiol.ogical Plant Pathology 9:77-86.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Molecular Plant-Microbe Interactions 8:863-870.

Leprince, O., Harren, F. J. M., Buitink, J., Alberda, M., and Hoekstra, F. A. (2000). Metabolic dysfunction and unabated respiration precede the loss of membrane integrity during dehydration germinating radicals. Plant Physiology 122: 597-608.

Levine, A., Tenhaken, R., Dixon, R. (1994).H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79:583-593.

Lund, S. T., Stall, R. E., and Klee, H. J. (1998). Ethylene regulates the susceptibility response to pathogen infection in tomato. The Plant Cell 10:371-382.

Malolepsza, U., and Urbanek, H. (2000). The oxidants and antioxidant enzymes in tomato leaves treated with o-hydroxyethylorutin and infected with Botrytis cinerea. European Journal of Plant Pathology 106:657–665.

Marte, M., Buonaurio, R., and Dellatorre, G. (1993). Induction of systemic resistance to tobacco powdery mildrew by tobacco mosaic virus, tobacco necrosis virus and ethephon. Journal of Phytopathology 138:137-144.

Mattoo, A. K., and Suttle, J. C. (1991). The plant hormone ethylene. CRC Press, Boca Raton, Fla.

Montero, C., Cristescu, S., Jimenez, J. B., Orea, J. M., Te Lintel Hekkert, S., Harren, F. J. M., and Gonzalez Urenia, A. (2003). trans-Resveratrol and grape disease resistance. A dynamical study by high-resolutiin laser-based techniques. Plant Physiology 131:129-138.

Montero, C., Bescós, B., Orea, J. M., and González Ureña, A. (2000). Food chemical analysis by laser desorption and resonant ionization mass spectrometry. Revista Analytical Chemistry 19:1-29.

Montero, C., Orea, J. M., Muñoz, M. S., Lobo, R. F., and González Ureña, A. (2000b). Non volatile analysis in fruits by laser resonant ionization spectrometry: Application to resveratrol in grapes. Applied Physics B 71:601-605.

Muckenschnabel I., Goodman B. A., Williamson B., Lyon G. D., and Deighton N. (2002). Infection of leaves of Arabidopsis thaliana by *Botrytis cinerea*; changes in ascorbic acid, free radicals, and lipid peroxidation products. Journal of Experimental Botany 53:207-214.

Mur, L. A. J., Santosa, I. E., Laarhoven, L. J. J., Holton, N. J., Harren, F. J. M., and Smith, A. R. (2005). Laser photoacoustic detection allows in planta detection of nitric oxide in tobacco following challenge with avirulent and virulent *Pseudomonas syringae* pathovars. Plant Physiology 138:1247-1258.

Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Tanase, S., Morino, Y., and Fukuda, H. (1991). Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2. Journal of General Microbiology 137:2281–2286.

Nazli, Z.-i.-H., Arshad, M., and Khalid, A. (2003). 2-Keto-4-methylthiobutyric acid-dependent biosynthesis of ethylene in soil. Biol. Fertil. Soils 37:130-135.

Oomens, J., Zuckermann, H., Persijn, S., Parker, D. H., and Harren, F. J. M. (1998). CO-laser based photoacoustic trace-gas detection: applications in postharvest physiology. Applied Physics B67:459-466.

Orea, J. M., Montero, C., Jiménez, J. B., and González Ureña, A. (2001). Analysis of *trans*-Resveratrol by laser desorption coupled with resonant ionization spectrometry. Application to *trans*-resveratrol content in vine leaves and grape skin. Analytical Chemistry 73:5921-5929.

Palou, L., Crisosto, C. H., Garner, D., Basinal, L. M., Smilanick, J. L., and Zoffoli, J. P. (2002). Minimum constant sulfur, dioxide emission rates to control gray mold of cold-stored table grapes. American Journal of Enol. Vitic. 53:110-115.

Palou, L., Crisosto, C. H., Garner, D., and Basinal, L. M. (2003). Effect of continuous exposure to exogenous ethylene during cold storage on postharvest decay development and quality attributes of stone fruits and table grapes. Postharvest Biology and Technology 27:243-254.

Parr, A. J. and Bolwell, G. P. (2000). Phenols in the plant and in the man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. Journal of Agricultural and Food Science 80:985-1012.

Patykowski J, and Urbanek H. (2003). Activity of enzymes related to H2O2 generation and metabolism in leaf apoplastic fraction of tomato leaves infected by *Botrytis cinerea.* Journal of Phytopathology 151:153-161.

Paul, B., Chereyathmanjiyil, A., Masih, I., Chapuis, L., and Benoit, A. (1998). Biological control of *Botrytis cinerea* causing grey mould disease of grapevine and elicitation of stilbene phytoalexin (resveratrol) by a soil bacterium. FEMS Microbiology Letters 165:65-70.

Penninckx, I. A. M., Eggermont, K., Terras, F. R. G., Thomma, B. P. H. J., de Samlanx, G. W., Buchala, A., Metraux, J. P., Manners, J. M., and Broekaert, W. F. (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell 8:2309-2323.

Petruzzelli, L, Harren F., Perrone, C., and Reuss, J. (1995). On the role of ethylene in seed germination and early root growth of *Pisum Sativum*. Journal of Plant Physiology 145:83-86.

Pezet, R., Pont, V., and Hoang-Van, K. (1991). Evidence for detoxification of pterostilbene and resveratrol by laccase-like stilbene oxidase produced by *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 39:441-450.

Pimentel, D., Culliney, T. W., and Bashore, T. (1996). Public health risks associated with pesticides and natural toxins in foods. In E.B. Radcliffe and W.D. Hutchison Eds. The Radcliffes's IPM World Textbook. University of Minnesota, St. Paul, MN.

Polevaya, Y., S. Alkalai-Tuvia, A. Copel, and E. Fallik. (2002). Early detection of grey mould development in tomato after harvest. Postharvest Biology and Technology 25:221-225.

Robison, M. M., Griffith, M., Pauls, K. P., and Glick, B. R. (2001). Dual role of ethylene in susceptibility of tomato to *Verticillium* wilt. Journal of Phytopathology 2:385–388.

Rodrigo, I., Vera, P., Tornero, P., Hernandez Yago, J., and Conejero, V. (1993). cDNA cloning of voroid-induced tomato pathogenesis-related protein P23: characterization as a vacuolar antifungal factor. Plant Physiology 102:939-945.

Roze, L. V., Calvo, A. M., Gunterus, A., Beaudry, R., Kall, M., and Linz, J.E. (2004). Ethylene Modulates Development and Toxin Biosynthesis in *Aspergillus* possibly via an ethylene sensor-mediated signaling pathway. Journal of Food Protection 67:438-447.

Saltveit, M.E. (1999). Effect of ethylene on quality of fresh fruits and vegetables. Postharvest Biology and Technology 15:279-292.

Santosa, I. E., Laarhoven L. J. J., Harbinson J., Driscoll S., and Harren F. J. M. (2003). Laser-based trace gas detection of ethane as a result of photo-oxidative damage in chilled cucumber leaves. Review of Scientific Instruments 74:680-683.

Sarig, P., Zutkhi, Y., Monjauze, A., Lisker, N., and BenArie, R. (1997). Phytoalexin elicitation in grape berries and their susceptibility to *Rhizopus stolonifer*. Physiological and Molecular Plant Pathology 50:337-347.

Schouten, A., Tenberge, K. B., Vermeer, J., Stewart, J., Wagemakers, C. A. M., Williamson, B., and van Kan, J. A. L. (2002a). Functional analysis of an extracellular catalase of *Botrytis cinerea.* Molecular Plant Pathology 3, 227-238.

Schouten, A., Wagemakers, L., Stefanato, F. L., van der Kaaij, R. M., and van Kan J. A. L. (2002b). Resveratrol acts as a natural profungicide and induces self-intoxication by a specific laccase. Molecular Microbiology 43:883-894.

Staal, M., te Lintel Hekkert, S., Harren, F., and Stal, L. J. (2001). Nitrogenase activity in cyanobacteria measured by the acetylene reduction assay: a comparison between batch incubation and on-line monitoring. Environmental Microbiology 3:343-351.

Staal, M., te Lintel-Hekkert, S., Harren, F., and Stal, L. J. (2003). Light action spectra of nitrogenase activity in Baltic Sea cyanobacteria. Journal of Phycology 39:668-677.

Stein, U., and Blaich, R. (1985). Investigations on the production of stilbenes and susceptibility to *Botrytis* of Vitis spp. Vitis 24:75-87.

Swirsky Gold, L., Slone, T. H., and Ames, B. N. (1997). Priorization of possible carcinogenic hazards in Food. In D.R. Tennant Ed. Food Chemical Risk Analysis, Chapman and Hall., New York, pp. 267-295.

Qadir, A., Hewett, E. W., and Long, P. G. (1997). Ethylene production by *Botrytis cinerea*. Postharvest Biology and Technology 11:85-91.

Qutob, D., Kamoun, S., and Gijzen, M. (2002). Expression of *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. Plant Journal 32:361–373.

Tadege, M., and Kuhlemeier, C. (1997). Aerobic fermentation duirng tabacco pollen development. Plant Molecular Biology 35:343:354.

te Lintel Hekkert, S., Staal, M. J., Nabben, R. H. M., Zuckermann, H., Persijn, S., Stal, L. J., Voesenek, L. A. C. J., Harren, F. J. M., Reuss, J., and Parker, D.H. (1998). Laser photoacoustic trace gas detection, an extremely sensitive technique applied in biological research. Instrum. Sci. Technol. 26:157-175.

ten Have, A., Breuil, W. O., Wubben, J. P., Visser, J., and van Kan, J. A. L. (2001). *Botrytis cinerea* endopoligalacturonase genes are differentially expressed in various plant tissues. Fungal Genetics and Biology 33:97-105.

Thain, S. C., Laarhoven, L. J. J., Dowson-Day, M. J., Wang, Z. Y., Tobin, E. M., Harren, F. J. M., van der Straeten, D., and Millar, A. J. (2004). Circadian rhythms of ethylene emission in Arabidopsis detected by laser photoacoustics. Plant Physiology 136:3751-3761.

Thomma, B., Eggermont, K., Tierens, K. F., and Broekaert, W. F. (1999).Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiology 121:1093-1102.

Thomma, B., Penninckx, I., Broekaert, W. F., and Cammue, B. P. A. (2001). The complexity of disease signaling in Arabidopsis. Current Opinion. in Immunology 13:63-68.

Thuring J. W. J. F., Harren F. J. M., Nefkens G. H. L., Reuss, J., Titulaer, G. T. M., de Vries, H. S. M., and Zwanenburg, B. (1994). Ethene production by seeds of Striga hermonthica induced by germination stimulants. In "Biology and management of Orobanche" (Eds.) A.H. Pieterse, J.A.C. Verkleij and S.J. ter Borg, Royal Tropical Institute, Amsterdam: 225-236.

Tomas, S. A., and Harren, F. J. M. (2001). Kinetics of ethanol and acetaldehyde production in fermenting wheat dough by laser-based trace gas detection. Food Science and Technology International 7:307-315.

van den Bussche, F., Smalle, J., Jie, L., Madeira Saibo, N. J., de Paepe, A., Chaerle, L., Tietz, O., Smets, R., Laarhoven, L. J. J., Harren, F. J. M., van Onckelen, H., Palme, K., Verbelen, J. P., van der Straeten, D. (2003). The *Arabidopsis* mutant *alh1* illustrates a cross talk between ethene and auxin. Plant Physiol.131:1228-1238.

van Kan, J. A. L., Cozijnsen, T., Danhash, N., and de Wit, P. J. G. M. (1995). Induction of tomato stress protein mRNAs by ethephon, 2,6-dichloroisonicotic acid and salicylate. Plant Molecular Biology 27:1205-1213.

van Loon, L. C. (1984). Regulation of pathogenesis and symptom expression in diseased plants by ethylene. In: Fuchs Y., Chalutz, E. editors. Ethylene: biochemical, physiological and applied aspects. The Hague: martinus Nijhoff/Dr. W.Junk, p. 171-180.

Veltman, R. H., Sanders, M. G., Persijn, S. T., Peppelenbos H. V., and Oosterhaven, J. (1999). Decreased ascorbic acid levels and brown core development in pears (*Pyrus communis* L. cv. Conference). Physiologia Plantarum 107:39-45.

Visser, E. J. W., Nabben, R. H. M., Blom, C. W. P. M.. and Voesenek, L. A. C. J. (1997). Growth of primary lateral roots and adventitious roots during conditions of hypoxia and high ethylene concentrations. Plant, Cell and Environment 20:647-653.

Voesenek, L. A. C. J., Banga, M., Thier, R. H., Mudde, C. M., Harren, F. J. M., Barendse, G. W. M., and Blom, C. W. P. M. (1993). Submergence induced ethylene synthesis, entrapment and growth in two plant species with a contrasting flooding resistance. Plant Physiology 103:783-791.

von Tiedemann, A. (1997). Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. Physiological and Molecular Plant Pathology 50:151-166.

Yang, S. F, and N. E. Hoffman. (1984). Ethylene biosynthesis and its regulation in higher plants. Annual Review of Plant Physiology 35:155-189.

Zuckermann, H., Staal, M., Stal, L. J., Reuss, J., te Lintel Hekkert, S., Harren, F. J. M., and Parker, D. H. (1997a). On line monitoring of nitrogenase activity in cyanobacteria by sensitive laser photoacoustic detection of ethylene. Applied and Environmental Microbiology 63:4243-4251.

Zuckermann, H, Harren, F. J. M., Reuss, J., and Parker, D. H. (1997b). Plant Physiology 113:925-932.

Wagstaff, C., Chanasut, U., Harren, F. J. M., Laarhoven, L. J., Thomas, B., Rogers, H. J., and Stead, A. D. (2005). Ethylene and flower longevity in *Alstromeria*: relationship between tepal senescence, abscission and ethylene biosynthesis. Journal of Experimental Botany 56:1007-1016.

Wang, J-Y., Cai, Y., Gou, J-Y., Mao, Y-B., XU, Y-H., Jiang, W-H., and Chen, X-Y. (2004). Applied and Environmental Microbiology 70:4989-4995.

Woltering, E. J., van Hout, M., Somhorst, D., and Harren, F. (1993). Roles of pollination and short-chain saturated fatty acids in flower senescence. Plant Growth Regulation 12:1-10.

Wubben, M. J. E, Su, H., Rodermel, S. R., and Baum, T. J. (2001). Susceptibility to the sugar beet cyst nematode is modulated by ethylene signal transduction in *Arabidopsis thaliana.* Molecular Plant-Microbe Interactiona 14:1206-1212.

Wubben, J. P., ten Have, A., van Kan, J. A. L., Visser, J. (2000). Regulation of endopolygalac-turonase genes expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. Current Genetics 37:152-157.