Natural extracts from pepper, wild rue and clove can activate defenses against pathogens in tomato plants

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Abbreviations: BABA, β-aminobutirric acid; BTH, benzo (1,2,3) thiadiazole-7-carbothioic acid; GUS, β-glucuronidase; INA, 2,6-dichloroisonicotinic acid; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; NaDC, sodium deoxycholate; PR, pathogenesis-related proteins; RT-qPCR, reverse transcription quantitative PCR; SA, salicylic acid; WT, wild type.

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Abstract

Tomato is an important species grown in many countries, either in fields or greenhouses. Despite decades of improvement, it is still susceptible to diseases, thus requiring the use of chemical pesticides, especially in greenhouses. Nevertheless it is imperative to reduce the use of environmental-unfriendly phytochemicals and favor less toxic tools to fight pathogens. Plants possess elaborate mechanisms against diseases that can lead to resistance. In the present work, we investigate the induction of plant defenses by means of extracts from plants widespread and easy to find, also known for their antimicrobial properties. Aqueous extracts of pepper 'Rojoto', wild rue and ethanolic extracts of clove powder (whose inhibiting effect was assessed on *Oidium* sp. spores) were tested on tomato plants for their ability to induce expression of different defense genes (PRs and regulatory proteins) after spraying. As revealed by RT-qPCR, all extracts were able to induce mRNA accumulation of different PR and MAPK regulators for several hours upon treatment, with clove and wild rue being the strongest. This effect could also be reproduced in tomato plants after a second treatment, 15 days after the first. The same extracts were tested in tomato and tobacco plants via leaf infiltration, showing necrotic symptoms associated with the hypersensitive response, thus confirming the priming capacity of the extracts. The involvement of

salicylic acid (SA) in these responses was verified by HPLC analysis and in SA-depleted transgenic tobacco (NahG). The results obtained suggest that natural antimicrobial extracts can be used to induce plant defenses and protect valuable crops. At the same time these low-cost extracts do not pose a threat to the environment or the farmer and can help reduce the farming costs, especially in developing countries.

Introduction

Tomato, a fruit generally treated as a vegetable, is a popular food and an important source of vitamins and antioxidants in the human diet. It is rich in lycopene, a pigment responsible for its characteristic red color, which is a very powerful antioxidant and it has been shown that can prevent and even fight cancer (Jaramillo et al. 2007).

Table tomato (*Solanum lycopersicum*), one of the most widely cultivated vegetable worldwide, represents a high percentage of commercial plants grown in Ecuador. In 2011 the harvested area was around 3,000 hectares with a production of more than 62,000 tons (INEC 2012), distributed mainly in the Sierra region. Almost all of this production is carried out with modern agriculture practices with the use of fertilizers and pesticides. This is due to the fact that production is mainly performed in greenhouses ensuring high and continuous production throughout the whole year (Jaramillo et al. 2007). The main disadvantage of greenhouse cultivation is the highest incidence of pest and diseases since humidity and temperature conditions are very favorable for their development.

One of the most common methods used to counteract these epidemics is the use of agrochemicals. These products play a major role in reducing economic damage to crops. The problem, however, is that these substances are characterized by high toxicity, persistence in the environment and misuse, therefore a rethinking of pest control strategies is needed (Seiber et al. 2014; Jeschke 2016). It is also true that the application of agrochemicals has replaced the ancestral use of plant extracts to fight diseases.

Research in agriculture and biology has been directed to the identification of new weapons to combat plant diseases, new tools that have a low environmental impact while still being effective against diseases (see European Directive 2009/128/EC). One alternative would be the use of molecules capable of activating the endogenous plant defenses, a strategy that has received much attention in recent years (Chaturvedi et al. 2012).

Plant defenses against biotic stresses are quite articulated and elaborate: besides constitutive defenses, plants can counteract pathogens attack via inducible responses (Frost et al. 2008). These require a recognition phase and a multileveled defensive phase (Dangl et al. 2013) that can result

in resistance. These responses require the participation of phytohormones, mainly salicylic acid (SA) and jasmonic acid (JA) (Dangl et al. 2013), small signaling molecules, proteins, enzymes and defensive molecules (for example: reactive oxygen species, the cell wall component callose and pathogenesis-related proteins, PR; Borges and Sandalio 2015; Dangl et al. 2013). The latter form a heterogeneous group of proteins (17 classes) with different functions and structures (Sels et al. 2008). In particular, PR1, despite its unknown function (probably antifungal, van Kan et al. 1992), is usually considered an efficient marker for resistance and its expression is induced by diseases and SA (Mitsuhara et al. 2008). In tomato plants, two PR1-enconding genes have been identified, basic PR1b1 and acidic PR1a2 (Tornero et al. 1997), whose pathogen- and hormone-dependent expression varies considerably, being PR1b1 the only one activated by pathogens and hormones (SA and ethylene, Tornero et al. 1997). PR2 refers to a group of ß-1,3-glucanases able to degrade the pathogen cell walls, while PR3 and 4 code for chitinases whose action against the chitin wall can inhibit pathogen growth (Van Loon et al. 1999). In tomato basic glucanases (PR2) (van Kan et al. 1992) have been shown to be rapidly activated by infection, as well as chitinases (PR3, Danhash et al. 1993). In terms of regulatory mechanisms, there is a growing evidence showing that mitogen-activated protein kinase (MAPK) play an early and crucial role in signaling the activation of plant defenses against pathogens (Meng and Zhang 2013). In particular in tomato two members of the MAPK gene family have been shown to be involved in pathogen resistance and defense gene activation: tomato gene MAPK2 (Stulemeijer et al. 2007) and MAPK7 (Kong et al. 2012). They appear to be associated with plant defense and pathogen infection and are closely related to Arabidopsis MPK3 and 6 proteins, which regulate immunity to pathogens (Nakagami et al. 2005).

It is known that various types of molecules may have a protective function against plant pathogens by stimulating endogenous mechanisms of resistance (Chaturvedi et al. 2012; Shah et al. 2014). Salicylic acid is one of these stimulating molecules: its application activates the resistance pathway which will protect the plant against pathogens (priming). However, other molecules have been shown able to induce priming defenses in plants (Conrath et al. 2015), including β-aminobutirric acid [BABA] (Baccelli and Mauch-Mani 2016), SA synthetic analogs such as 2,6-dichloroisonicotinic acid [INA] (Kessman et al. 1994) and benzo (1,2,3) thiadiazole-7-carbothioic acid [BTH] (Görlach et al. 1996). Unfortunately these inducers have not been significantly applied in the field due to undesired loss of fitness and to a limited effect - protective rather than curative, thus requiring pretreatment (Conrath 2009).

Recently it has been shown that other molecules can indeed activate plant defenses in a similar fashion, helping plants fight pathogens. These molecules do possess a further characteristic as compared to BABA, INA or BTH: they are known for their antimicrobial properties, therefore they combine a negative effect on pathogen growth (curative) and the activation of the plant defensive mechanisms (protective). For example, the strobilurin fungicide Pyraclostrobin was shown to induce a strong accumulation of PR1 during pathogen attack in tobacco plants (Herms et al. 2002). More recently two other molecules, not pertaining to the plant pesticide category, have been shown able to activate plant defenses and plant defense-related genes. Near-neutral solutions of hypochlorous acid (obtained by electrochemical activation of a KCl diluted solution, Zarattini et al.

2015) and of the bile acid deoxycholate (Zarattini et al. 2016), both well known for their antimicrobial and disinfectant properties, were shown to activate plant defenses and several PR proteins after one or two treatments in tobacco, Arabidopsis and apple plants. Repeated treatment in apple orchard with the electrochemical activated solution (ECAS) did exert a therapeutic effect on trees infected with *Nectria galligena*, without any visible negative side effects (Zarattini et al. 2015). On the other hand, the sodium deoxycholate solution could induce several defense-related genes as shown by microarray analysis and could interfere with phytopathogenic bacterial growth in leaves (Zarattini et al. 2016). Both these examples show that it is possible to combine in the same solution an antimicrobial property and a defense-activating effect, thus fighting the pathogen both from the outside and the inside of the plant. Furthermore these examples reveal that it is possible to use environmental friendly molecules instead of agrochemicals, thus avoiding the negative impact, which is unfortunately common with synthetic pesticides.

Aim of the present work was therefore to establish if it is possible to find properties similar to ECAS, deoxycholate or Pyraclostrobin also in natural extracts obtained from plants known for their antimicrobial properties, through popular knowledge and scientific investigation. Therefore we chose to test several natural extracts obtained from plants commonly found to establish if they are able to activate plant defenses by means of PR genes activation and hypersensitive response. The model plant chosen was tomato for its agronomic importance and economical value in Ecuador as well as in other countries. The analysis allowed to assign to chilli pepper, rue and clove extracts the capacity to induce plant defensive proteins and responses as judged by different experimental evidence. These plants are quite well known for their properties in natural medicine, but also for their antimicrobial activity (Dorantes et al. 2000; Careaga et al. 2003; Sarpeleh et al. 2009; Pandey and Singh 2011; Al-Ani et al. 2012; Moloudizargari et al. 2013; Saeidi et al. 2015). Here we describe that simple aqueous or ethanolic extracts can indeed activate plant defensive proteins in tomato (and tobacco) plants and therefore could act as natural pesticides to be used in the field.

Materials and methods

Plant materials

Tomato plants (*Solanum lycopersicum* L) and tobacco (*Nicotiana tabacum* cv. Petite Havana SR-1) were grown in a controlled growth chamber with a 16/8 h photoperiod at 24 °C and 60% of humidity. The tobacco transgenic plants NahG (Friedrich et al. 1995; kindly provided by Dr. Luis Mur, University of Aberystwyth, Wales, UK) and PR1a-GUS (Grüner and Pfitzner 1994; kindly provided by Dr. Ursula Pfitzner, University of Hohenheim, Stuttgart, Germany) were grown in the same conditions.

Plant extracts preparation

Fresh red chilli peppers (*Capsicum annum*, var. Rojoto), freshly-cut wild rue plants (*Peganum harmala*) and clove powder (*Syzygium aromaticum*) where purchased in local markets and used for the preparation of plant extracts. Aqueous extracts were prepared similarly to Sarpeleh et al. (2009). Briefly, pepper (25g) and wild rue plants (12g) where cut in small pieces and submerged in 100 mL of water for 16h in the darkness, filtered and used without dilution. Clove ethanolic extracts were prepared according to Pandey and Singh (2011) and used at a final concentration of 1% (v/v) in water. Before plant treatments, the different solutions were prepared with the addition of a wetting agent (Agrotafix, containing polyethoxylates, 30ml/L) and the pH titred to 6.5. Control treatments were performed with water alone (pH 6.5; negative control), diluted hypochlorous acid (250mg/L, pH 6.5) and the bile acid sodium deoxycholate (200µM, NaDC, pH 6.5) as positive controls (Zarattini et al. 2015 and 2016, respectively).

Treatments and sampling

The different solutions were sprayed on tomato or tobacco plants (2-months old) until their complete wetting. 24 and 48h after the first treatment, two-three leaves from two to three plants were harvested and immediately frozen in liquid nitrogen. A second treatment was performed on the same plants 15 days after the first and leaf samples were collected after 24 h and 48 h. Leaf samples were then grounded in liquid nitrogen and stored at -80 °C.

RNA extraction and reverse transcription quantitative PCR

Total RNA was extracted from 100 mg of powdered leaves by “Spectrum™ Plant Total RNA Kit” (Sigma). DNAse treatment (Sigma) was carried out as suggested by the manufacturer. The relative quantification of mRNAs was performed by two steps reverse transcription quantitative PCR analysis. In the first step, single-strand cDNA was synthesized with the Transcriptor Universal cDNA Master Kit (Roche) following the manufacturer’s instructions. After a 1:10 dilution, cDNA was then subjected to qPCR in the presence of SYBR green (FastStart DNA Green Master Mix, Roche) on a LightCycler® Nano Instrument (Roche). The genes analysed in tomato and tobacco plants are listed in Table 1.

Table 1. Defense-related genes analyzed by RT-qPCR, for both tomato and tobacco plants, with their GenBank accession numbers.

Gene	Function	Tomato	Tobacco
PR1	Antifungal?	Y08804 (Pr1b1)	X06361 (PR1a)
PR2	β-1,3-glucanase	M80608	M60460
PR3	Acidic chitinase	Z15141	X51426
MAPK2	MAP Kinase	NM_001247426	
MAPK7	MAP Kinase	NM_001246968	
Actin	Reference gene	U60481	U60493
EF1α	Reference gene	NM_001247106	AF120093

The relative expression levels of all genes were calculated using the geNorm algorithm (Vandesompele et al. 2002). This algorithm requires the use of two stable reference genes (in our case Actin and Elongation Factor 1α) and the comparison between the treated and control samples. RT-qPCR reactions were performed in duplicate for at least three independent treatments. The results obtained have been expressed as "fold change", i.e. the over-expression of the genes in treated samples as compared to the negative control sample.

SA quantification by HPLC

To determine the amount of salicylic acid in tomato leaves, 50 mg of leaf tissue were ground in liquid nitrogen and extracted with 1 mL of extraction mixture (10% methanol, 1% acetic acid, 89% water), vortexed for 30 seconds and degassed for 5 minutes. After centrifugation at 13000g for 10 minutes, the supernatant was recovered in a fresh tube. The extraction procedure was then repeated once again with 500 µL of extraction mixture. After filtration through a 0,45µm filter, the samples were subjected to HPLC analysis. Quantification of SA was performed in triplicates in a Waters 1525 HPLC with PDA detector, equipped with a XBridge C-18 column 4.6 x 150 mm at 36.5°C. Mobile phases were 1:1 v/v Acetonitrile-Water with Formic Acid 0.1% v/v, at isocratic mode, flow rate 0.6 mL/min. Detection wavelength 296 nm. The injection volume was 10 µL, run time 4 minutes. Salicylic peak appeared at retention times of 2.5 minutes. SA amount was expressed as µg/g fresh weight, mean values were obtained from 3 replicas and analysed by ANOVA.

***Oidium* sp. germination assay**

For this assay 100 uL of the plant extracts were filtered with 0.45µm PVDF filter and placed in a 96 well plate microtiter following the double dilution method with water. Diluted (250mg/L, pH 6.5) hypochlorous acid was used as positive and water as negative control. Spores of the powdery

mildew fungus *Oidium* sp. were obtained from contaminated plants, by washing the leaves in water. They were then resuspended in PDB medium at a final concentration of $1x10^8$/mL, measured in a Neubauer chamber. 100 µL of spores were then added to each sample, which was run in duplicate. The spores were incubated at 23°C for 7 days and the germination efficiency was assessed daily by microscopic analysis.

***In vivo* leaf infiltration and GUS histochemical analysis**

Tomato and tobacco wild type leaves were infiltrated with different solutions (see above) according to Noda et al. (2010) and Benouaret et al. (2014) and photographed 24 and 48 hours after treatment. PR1a-GUS transgenic plants treated with different solutions (BTH, 140mg/L was used as positive control) were assayed for GUS activity by histochemical assay carried out with a modified protocol (Degrave et al. 2008). Briefly, tobacco leaves were harvested 24 and 48 h after the treatment and incubated in 50 mM Na_2HPO_4 and 10 mM EDTA, pH 7.0 containing the X-gluc substrate (0.05% w/v). Staining was performed in darkness at 37 °C for 16-20 h. To remove chlorophyll, leaves were washed several times with hot 70% ethanol.

Results and discussion

The research work was focused on the fight against diseases in plants of agricultural interest using natural products with low environmental impact, able to exert an antimicrobial action and, at the same time, to elicit plant endogenous defenses. Plant extracts already known in natural medicine for antimicrobial effects were tested in a plant species of great agronomic interest such as tomato (native species in Latin America) in a particularly difficult environment for infections (humidity, altitude, latitude). The project aims to improve the beneficial effects of extracts of local plants from Ecuador as stimulant of plant defenses: this option would have a positive impact on the protection of biodiversity and on the local economy (reduced use of expensive agrochemicals and the possibility of local entrepreneurship).

Healthy tomato plants were treated with different aqueous or ethanolic extracts obtained from different plants easily found in local markets: red chilli pepper 'Rojoto', wild rue, the field horsetail (*Equisetum arvense*), common wormwood (*Artemisia vulgaris*), garlic (*Allium sativum*) and dried clove powder. 24 and 48h after treatment leaf samples were collected and use to establish RNA expression levels of several PR-encoding genes considered as markers for plant disease resistance (van Kan et al. 1992). As a preliminary screening, cDNAs derived from total RNA extracted from the different leaf samples were subjected to RT-qPCR amplification using primers directed to a major PR-coding gene (PR1). Primers for the reference genes Actin and EF1α were used for normalization. After relative quantification, by the geNorm algorithm (Vandesompele et al. 2002) performed against water-treated plants (negative controls), we were able to eliminate some extracts (horsetail, garlic, wormwood; data not shown) therefore focusing on pepper, rue and

clove only. The antimicrobial effect of these three extracts, already known though the literature available, was further confirmed by an *in vitro* bioassay on *Oidium* sp. spore germination. The three extracts and their dilutions were tested for their ability to inhibit spore germination from field-collected *Oidium* sp. As shown in Table 2, the inhibitory effect is seen with the undiluted extracts while it rapidly disappears with further dilutions. This inhibitory effect appeared comparable, even if weaker, to the one exerted by near-neutral hypochlorous acid (250mg/L) as positive control. This confirmed the indication obtained in previous publications (Dorantes et al. 2000; Careaga et al. 2003; Sarpeleh et al. 2009; Pandey and Singh 2011; Al-Ani et al. 2012; Moloudizargari et al. 2013; Saeidi et al. 2015).

Table 2. Inhibitory effect of plant extracts on the germination of *Oidium* sp. spores. Different concentrations (100%, undiluted; 50% and 25% dilutions in water) of the extracts were tested for their ability to inhibit spore germination. Active chlorine was used as positive control. Average percentage of germination, n = 2.

Concentration	H2O	HClO	Pepper	Rue	Clove
100%	+++	-	+/-	+/-	+/-
50%	+++	+/-	++	++	++
25%	+++	+	++	++	++
% of germination: - <10%; +/- 10-25%, + 25-50%, ++ 50-75%, +++ > 75%					

A much deeper expression analysis was then performed by RT-qPCR on tomato leaf samples collected from plants treated with the three natural extracts as well as with water (neg. contr.) and two positive controls (HClO and sodium deoxycholate, Zarattini et al. 2015 and 2016). Two timepoints were considered, based on previous works, 24 and 48 hours after treatment and the group of primers used contained genes involved in plant defenses against pathogens (PR1, 2 and 3, members of the PR family, and MAPK2 and 7, regulatory proteins, Table 1). The results from RT-qPCR allowed the comparison between the fold change observed in treated plants with that of control-treated plants. As shown in Figure 1, PR1 mRNA accumulated abundantly in tomato leaves after the treatment with pepper, rue or clove extracts (more than 100x for rue and clove), similarly to the levels obtained with the positive controls (HClO and NaDC). These elevated expression levels were maintained up to 48 h after each treatment. The expression level observed is quite high, in line with previous research in tobacco (Benouaret et al. 2014), Arabidopsis (Tuzun and Somanchi 2006) and several other species (Verhagen et al. 2006; Floryszak-Wieczorek et al. 2015) thus showing that PR1 can be rightly considered an informative molecular marker for plant defenses activation.

Figure 1

Similarly, RT-qPCR was used to follow the expression pattern of other PR genes, in particular, PR2 (glucanase) and PR3 (chitinase). PR2 gene was up-regulated as compared to water treated plants in a variable manner upon the different treatments (Figure 1). Pepper and wild rue only induced a moderate mRNA accumulation 24 hours after treatment, while the clove extract was more efficient, leading to an almost 10x increase in mRNA level. After 48h hours the expression level was still high for the clove-treated sample and still low for the wild rue one. On the contrary, pepper extract induced a 14x accumulation, as compared to the negative control. These induction levels are even higher than those observed for the two positive controls (HClO and NaDC). ß-1,3-glucanases are well known pathogenesis related proteins and their expression is induced by different kind of pathogens and defense-related phytohormones (Spoel and Dong 2012), furthermore transgenic plants studies have shown that overexpressing PR2 glucanases increased disease resistance and delayed symptoms (Balasubramanian et al. 2012). The natural extracts used in this work, being able to stimulate the accumulation of glucanases, are able to increase the defensive level of the treated plants before infection and therefore protect tomato plants against the biotic stress.

The analysis of chitinase-encoding PR3 gene expression profile furthermore revealed that pepper, wild rue and clove extracts are also able to induce chitinase accumulation 24h after treatment with a further increase in mRNA levels 48h after spraying, at levels similar to the positive controls (Figure 1). The simultaneous presence of both types of pathogen defense proteins has been already associated with increased resistance to diseases (Balasubramanian et al. 2012) and it is a common feature in primed plants and in systemic acquired resistance (Conrath 2009). It is also interesting to note that the different PR genes show also different sensitivity to phytohormones. For example, in Arabidopsis PR1 and PR2 are mainly activated through salicylic acid, while PR3 is more sensitive to jasmonic acid, (Spoel and Dong 2012), even if this is not always the case and there is also an overlapping between the responses mediated by the two hormones (Glazebrook, 2005). The plant extracts used in the present study appear therefore able to activate defense–related genes associated with both the SA and JA signaling pathways.

A further indication of the significant role of our plant extracts in activating plant defenses was obtained through the analysis of the expression profiles of two regulators of plant resistance belonging to the MAPK group. Tomato MAPK2 and MAPK7 were chosen due to their known role in the regulation of the hypersensitive and pathogens resistance (Stulemeijer et al. 2007; Kong et al. 2012). Both MAPK-encoding genes appeared to be activated (4-8x fold change, Figure 1) upon treatment with pepper, rue and clove extracts, MAPK2 being slightly more abundant as compared to MAPK7. The observed mRNA abundance did not reach the levels observed with the positive controls (up to 15x for NaDC) and appeared also time dependent, since no significant increase was

detected 48 h after treatment (data not shown). Therefore the regulatory proteins belonging to the MAPK group appeared rapidly and transiently up-regulated upon treatment.

Previous research work on tobacco and apple described an enhancement effect associated with the activation of plant defenses by active chlorine (Zarattini et al. 2015) upon repeated treatments. A second treatment 15 days after the first did result in a strong up-regulation of the PR genes analyzed. Therefore, the tomato plants of the first experiment were treated again with fresh pepper, rue and clove extracts, two weeks after the first treatment, RNA extracted after 24 and 48h and analyzed by RT-qPCR. The results shown in Figure 2 suggest the existence in tomato plants of an enhancement effect, though limited, as also seen in tobacco or apple plants. The plant extracts tested appeared to be able to up-regulate the defense genes upon treatment but the expression levels did not appear higher than the levels observed after the first treatment in the case of PR1, PR3 and MAPK2 (MAPK7 did not show any up-regulation, data not shown). PR2, on the other hand, did show a stronger activation upon a second treatment with wild rue and clove extracts. The PR2 expression levels (fold change) moved in fact from below 10x (Figure 1) up to 66x max after wild rue treatment (24h; Figure 2) or 54x for clove. We can therefore conclude that tomato plants, treated with plant extracts, activate defense genes transcription upon treatment for several hours and even after repeated treatments. This RNA accumulation pattern concerns both pathogenesis-related proteins as well as regulatory ones and was never accompanied by stress symptoms or a decrease in growth (data not shown).

Figure 2

To confirm the molecular data obtained with the natural extracts in tomato leaves, additional experimental tools were used. Firstly, PR1-GUS tobacco transgenic plants were grown and treated with the same natural extracts used on tomato plants. This model plant (Grüner and Pfitzner 1994) allows to monitor PR1 promoter activity by means of the GUS reporter gene, whose corresponding protein can be easily detected by a histochemical staining procedure. Tobacco healthy PR1-GUS plants were sprayed with pepper, wild rue and clove extracts in the same conditions used for tomato. 24 and 48h after treatment leaf samples were collected and subjected to X-gluc staining, specific for GUS activity. Upon discoloration, the blue staining was evaluated and photographed. Figure 3 show a representative image of the PR1-GUS activation profile observed in tobacco plants upon each treatment. GUS staining was observed for all extracts, 24 and 48 hours after a first treatment, being comparable to the staining observed in BTH treated tobacco plants (positive control). Water treated plants never showed any GUS activation (Figure 3). These observations are therefore in agreement with the data obtained in tomato leaves by RT-qPCR concerning the PR1 gene activation profile. They also suggest that the defense induction effect exerted by the three plant extracts in tomato leaves can be also reproduced in tobacco plants. The same plants were treated a second time with the same plant extracts, 15 days after the first treatment. As can be

seen in Figure 3 a second spraying with pepper and wild rue extracts induced a modest induction of the GUS transgene, while the clove extract was much more efficient causing a stronger staining, comparable to the BTH positive control. This is again in accordance with the previous molecular data which showed that a second treatment did induce defense gene activation, but at a lesser extent in the case of PR1. Taken together these results suggest that pepper, wild rue and clove extracts are able to activate plant defenses by simple leaf spraying without negative effects. Different PR-GUS transgenic plants have already been used as experimental tools to evaluate the capacity of induction of endogenous defenses and have proved to give useful indications (Herms et al. 2002; Conrath 2009).

Figure 3

Secondly, to evaluate if the three plant extracts are able to induce hypersensitive response, leaf infiltration experiments were performed in tomato as well in tobacco (model plant for infiltration experiments) plants. Healthy leaves were infiltrated with pepper, wild rue and clove extracts using a syringe and leaf phenotype was analyzed 1 or 2 days later. In tobacco leaves infiltrated with the plant extracts necrotic areas typical of the hypersensitive response (Figure 4) clearly appeared 24 and 48 hours after each treatment. The stronger response in terms of necrotic symptoms was observed with clove extract, in both timepoints. The whole infiltration area appeared necrotic and translucent due to cell death. Pepper and wild rue extracts did induce necrotic lesions, but at a lesser extent and especially at 48h. Similar responses were also observed with the control solutions (chlorine and NaDC, data not shown), but not with water alone. Similar infiltrations were performed in parallel in tomato leaves as well. Because tomato leaves are less prone to infiltration the results for pepper and wild rue were less evident in the 24h sample but still visible after 48h. Clove extract on the contrary induced clear necrotic symptoms (Figure 4) even in the 24h sample.

These experiments confirm the gene expression observations: pepper, wild rue and clove extracts identified by RT-qPCR were able to activate a local hypersensitive response along with cell death, a sign of the functional activation of the immune response with high efficiency. The infiltration experiment is significantly more aggressive on leaf tissue as compared to spraying: normal spraying did not induce any visible necrotic symptoms, but did indeed induce defense gene activation.

Figure 4

The mechanism of priming and activation of plant defenses against pathogens are known to exploit different plant hormones and signaling molecules (Denancé et al. 2013). Ethylene, jasmonic acid and salicylic acid play a central role in regulating the immune response of plants with SA being

a key regulator of plant resistance to pathogens and of systemic acquired resistance (SAR), a well-studied type of induced resistance (Glazebrook 2005).

Several defense genes have been shown to be dependent of SA (PR1 for example, Conrath 2009) therefore we tried to correlate the gene expression data with SA phytohormone quantity in treated tomato leaves. SA was extracted from different leaf samples treated with pepper, rue and clove extracts along with hypochlorous acid and water as controls, 24 and 48 hours after treatment. After clarification, SA amount was measured by HPLC. As shown in Figure 5, the amount of SA increased significantly 24h after treatment with the extracts as compared to the negative control, with a 1.5x fold change upon clove-treatment and a 1.8x fold change upon pepper treatment. These values are similar to the SA levels obtained after treatment with hypochlorous acid (2.4x). 48 hours after pepper, rue and clove treatment the SA amount measured was lower (fold changes about 1.3x-1.4x), still higher than the negative control but nevertheless not statistically different from the negative control. The positive HClO-treated sample showed a SA amount significantly higher than the negative control in both timepoints tested. SA increase upon treatment is therefore correlated with gene expression data, suggesting that SA accumulation could be responsible for the upregulation of at least some of the PR or MAPK genes studied.

Figure 5

To further verify the role of SA in the activation of genes that we observed in tomato and tobacco plants after treatment with various natural extracts, a transgenic model plant was employed. Tobacco plants expressing a salicylate hydroxylase (NahG, from *Pseudomonas*) gene convert salicylic acid to catechol, thus eliminating the hormone from plant tissues and creating a SA-depleted environment (Friedrich et al. 1995). Thanks to the similarities between tomato and tobacco, both Solanaceae, we can confidently draw a parallel between the observations made in tomato to those in tobacco. Therefore, WT and NahG tobacco plants were treated with the extracts from pepper, wild rue and cloves, RNA extracted after 24 hours and retrotranscribed. The expression levels of several tobacco defense genes were analyzed by RT-qPCR and compared to water treated controls. As shown in Figure 6, the natural extracts are capable of inducing the expression of PR1, PR2 and PR3 defense genes in WT tobacco plants, especially wild rue and clove (up to 44x fold change), in a similar fashion as observed in tomato. When the same treatments were performed on NahG transgenic plants without SA, induction of PR genes was completely abolished with pepper and clove extracts while with wild rue PR genes up-regulation appeared sensibly lower (PR2 10x fold change as compared to 44x in WT) than the one observed in WT control plants. These data suggest that the phenomenon of defense activation induced by pepper and cloves extracts requires unequivocally SA, since overexpression of defense genes is canceled in the absence of the hormone. On the other hand, this is only partially true for wild rue: in this

case the defense genes up-regulation is affected by the mutation but not completely, meaning that a SA-independent activation pathway is also evoked by wild rue extract.

Figure 6

In parallel, the same plant extracts were infiltrated in healthy NahG tobacco transgenic plants, depleted of SA. In this kind of mutant tissues, the hypersensitive-response symptoms appeared less strong and evident, with no differences between 24h (Figure 4) and the 48h (data not shown) timepoints. The translucent areas in the infiltrated leaves were less conspicuous and the necrotic symptoms were almost absent. These observations suggest furthermore that the defense activation obtained by treatment with pepper, wild rue and clove extracts do require SA (at least partially) to be elicited.

The observed activation induced by natural extracts is based on a complex interaction between different plant hormones, signaling molecules and regulatory pathways. A similar situation has been observed in other species treated with active chlorine (Zarattini et al. 2015). It is well known that different levels of pathogen defenses are elicited by different and overlapping pathways (Conrath 2009; Dangl et al. 2013) so it is not surprising that different plant extracts activate plant defenses in different fashions.

Conclusions

The data collected in this study reveal that natural extracts, obtained from pepper, wild rue and clove, when applied to tomato plants induced an increased level of transcription of defense and regulatory genes thus showing a priming effect. The plant extracts used in the present study appear also able to activate both SA-dependent and -independent signaling pathways and to induce necrotic, priming-related symptoms when infiltrated directly into leaves. These easy-to-make extracts are derived from plant material that can be easily found, with low costs and are well known for their antimicrobial action. Therefore these extracts combine several interesting features (antimicrobial effects, resistance-induction, inexistent toxicity for the environment) as revealed by molecular and other studies. They could therefore be used as phytoprotective agriculture agents in the fight against pathogens.

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Table 1. Defense-related genes analyzed by Real Time PCR, for both tomato and tobacco plants, with their GenBank accession numbers.

Gene	Function	Tomato	Tobacco
PR1	Antifungal?	Y08804 (Pr1b1)	X06361 (PR1a)
PR2	β-1,3-glucanase	M80608	M60460
PR3	Acidic chitinase	Z15141	X51426
MAPK2	MAP Kinase	NM_001247426	
MAPK7	MAP kinase	NM_001246968	
Actin	Reference gene	U60481	U60493
EF1α	Reference gene	NM_001247106	AF120093

Table 2. Inhibitory effect of plant extracts on the germination of *Oidium* sp. spores. Different concentrations (100%, undiluted; 50% and 25% dilutions in water) of the extracts were tested for their ability to inhibit spore germination. Active chlorine was used as positive control. Average percentage of germination, n = 2.

Concentration	H2O	HClO	Pepper	Rue	Clove
100%	+++	-	+/-	+/-	+/-
50%	+++	+/-	++	++	++
25%	+++	+	++	++	++
% of germination: - <10%; +/- 10-25%, + 25-50%, ++ 50-75%, +++ > 75%					

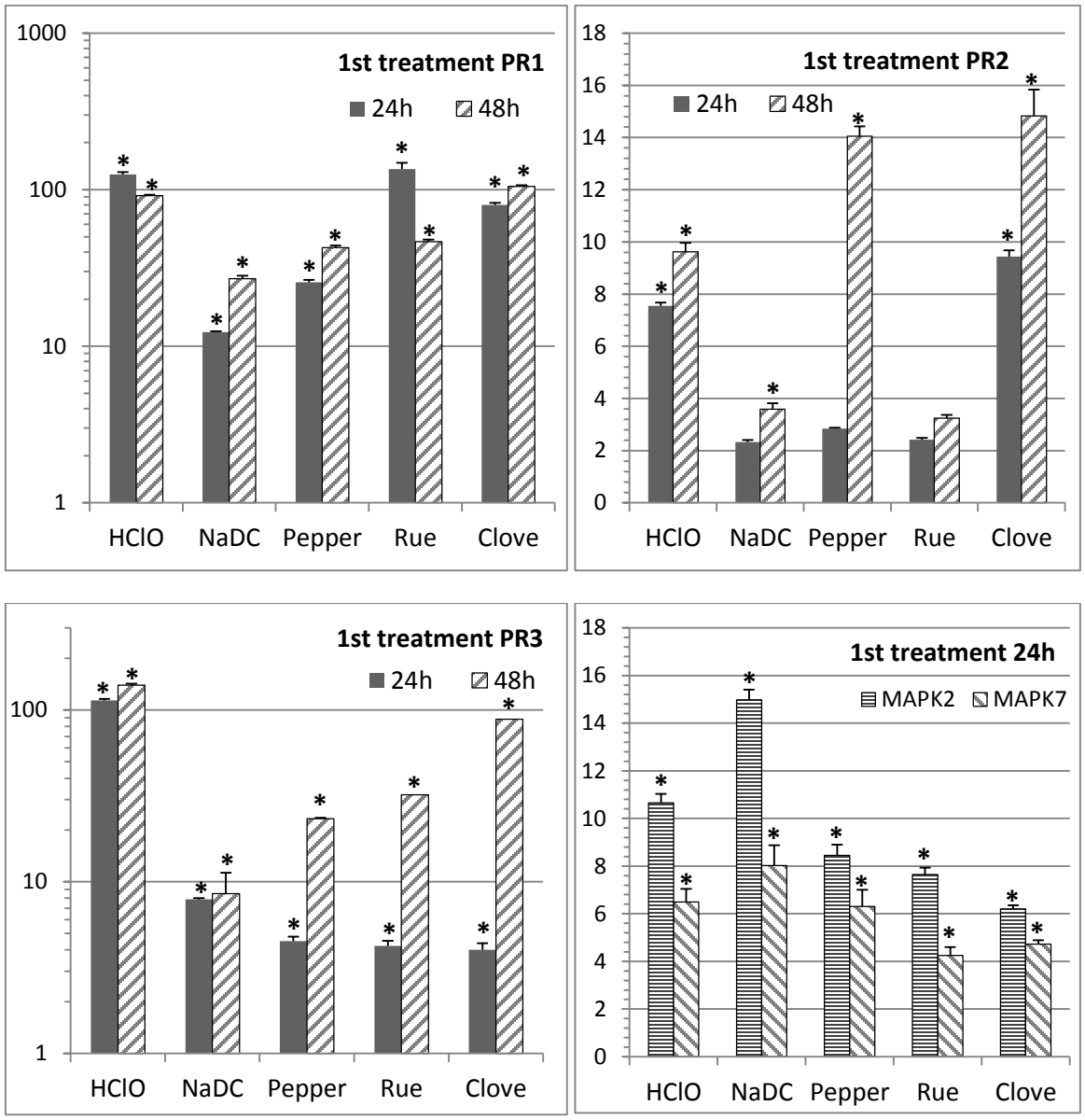


Figure 1 Levels of gene induction (fold change) of different defense genes (PR1, PR2, PR3, MAPK2 and MAPK7) in tomato leaves 24 and 48 hours after treatment with different plant extracts: pepper, wild rue and clove. The expression level obtained by RT-qPCR was compared to the mRNA level in negative control plants (treated with water) using the GeNorm algorithm. Active chlorine (HClO) and sodium deoxycholate (NaDC) were used as positive controls. Average fold change +SD, n = 3. The star (*) denotes a value significantly different from the negative control (water) by t-Student test.

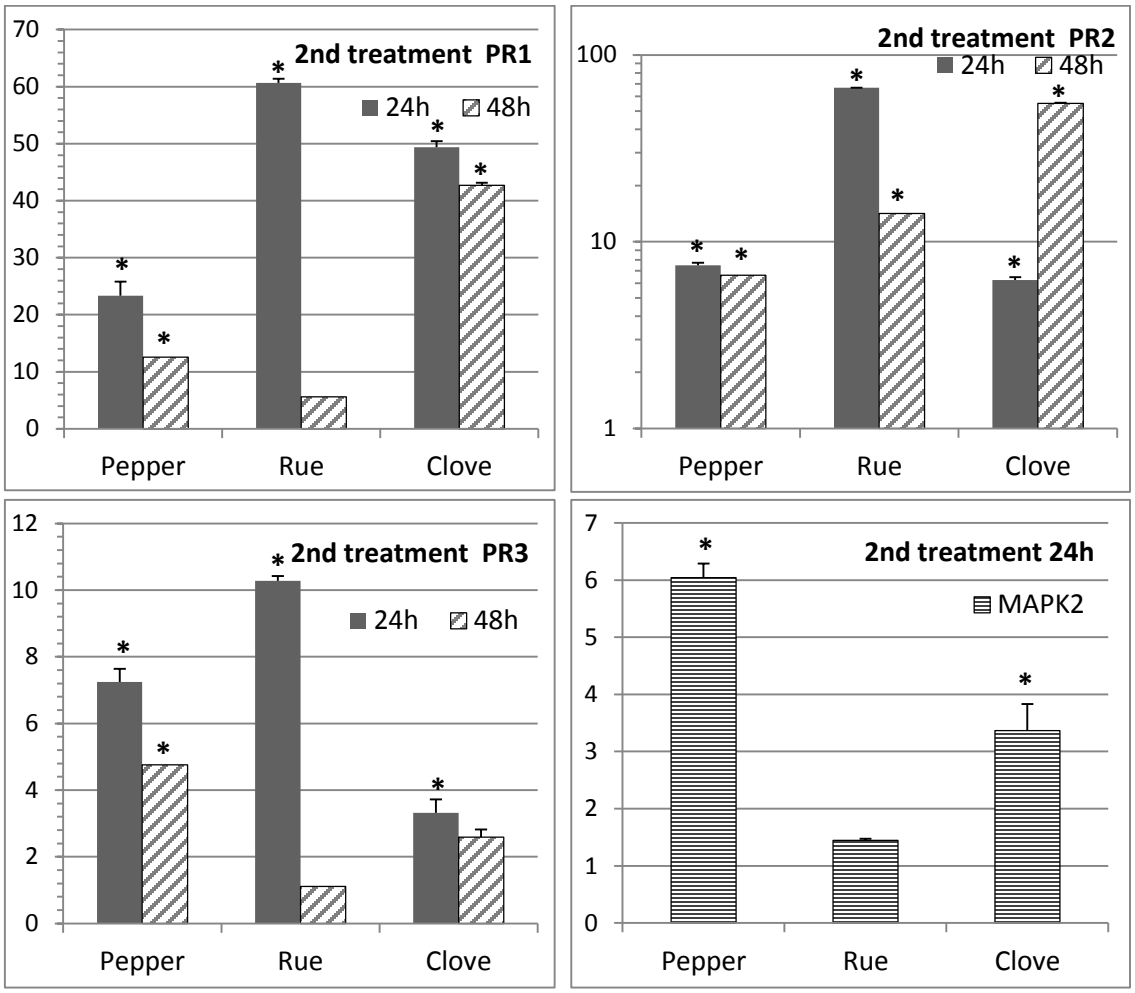


Figure 2 Levels of gene induction (fold change) of different defense genes (PR1, PR2, PR3 and MAPK2) in tomato leaves 24 and 48 hours after a second treatment (15 days after the first) with different plant extracts: pepper, wild rue and clove. The expression level obtained by RT-qPCR was compared to the mRNA level in negative control plants (treated with water) using the GeNorm algorithm. Average fold change +SD, n = 3. The star (*) denotes a value significantly different from the negative control (water) by t-Student test.

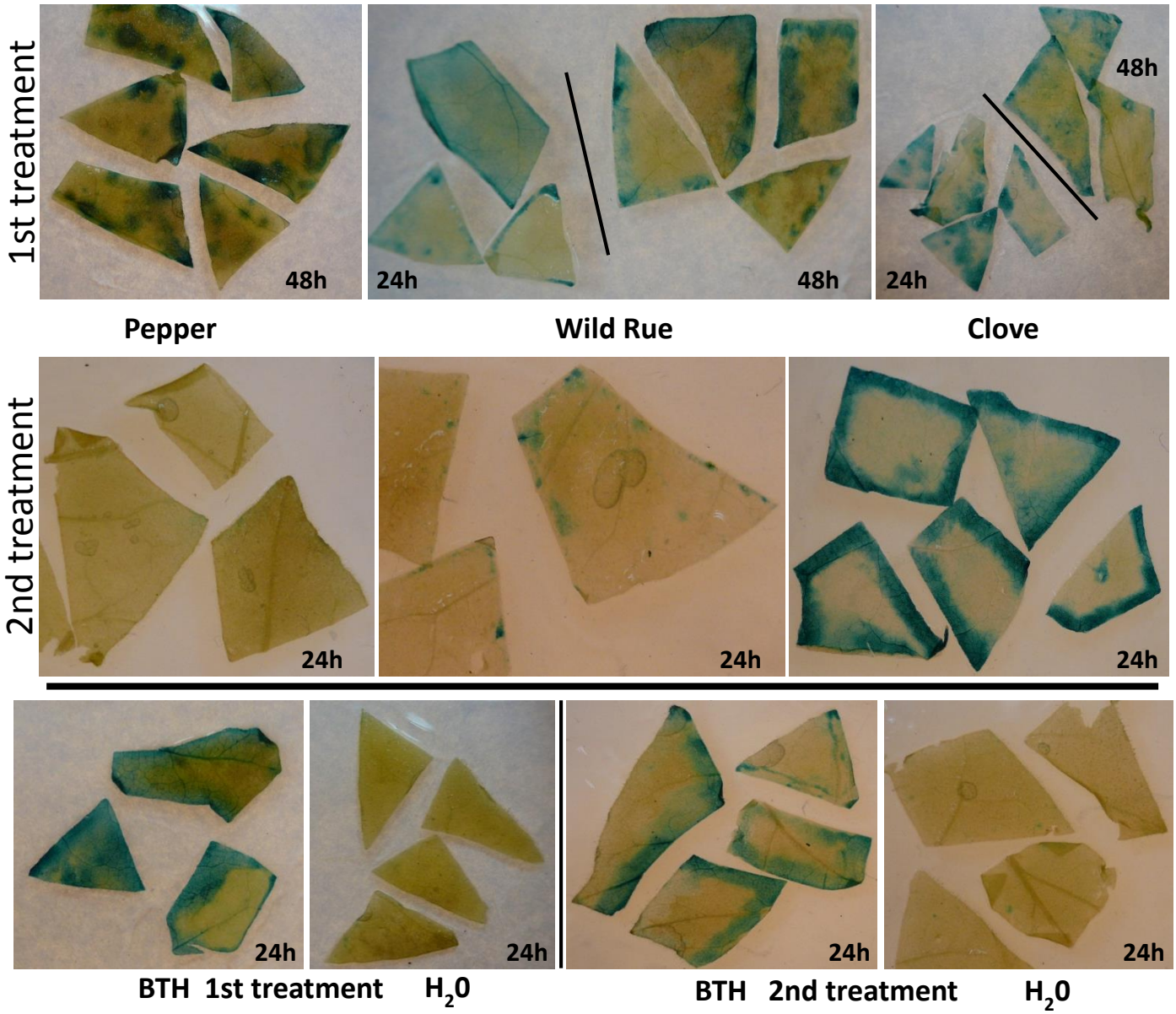


Figure 3 GUS histochemical staining on PR1a-GUS transgenic tobacco plants, 24 or 48 hours after different treatments with pepper, wild rue and clove extracts. BTH was used as positive control.

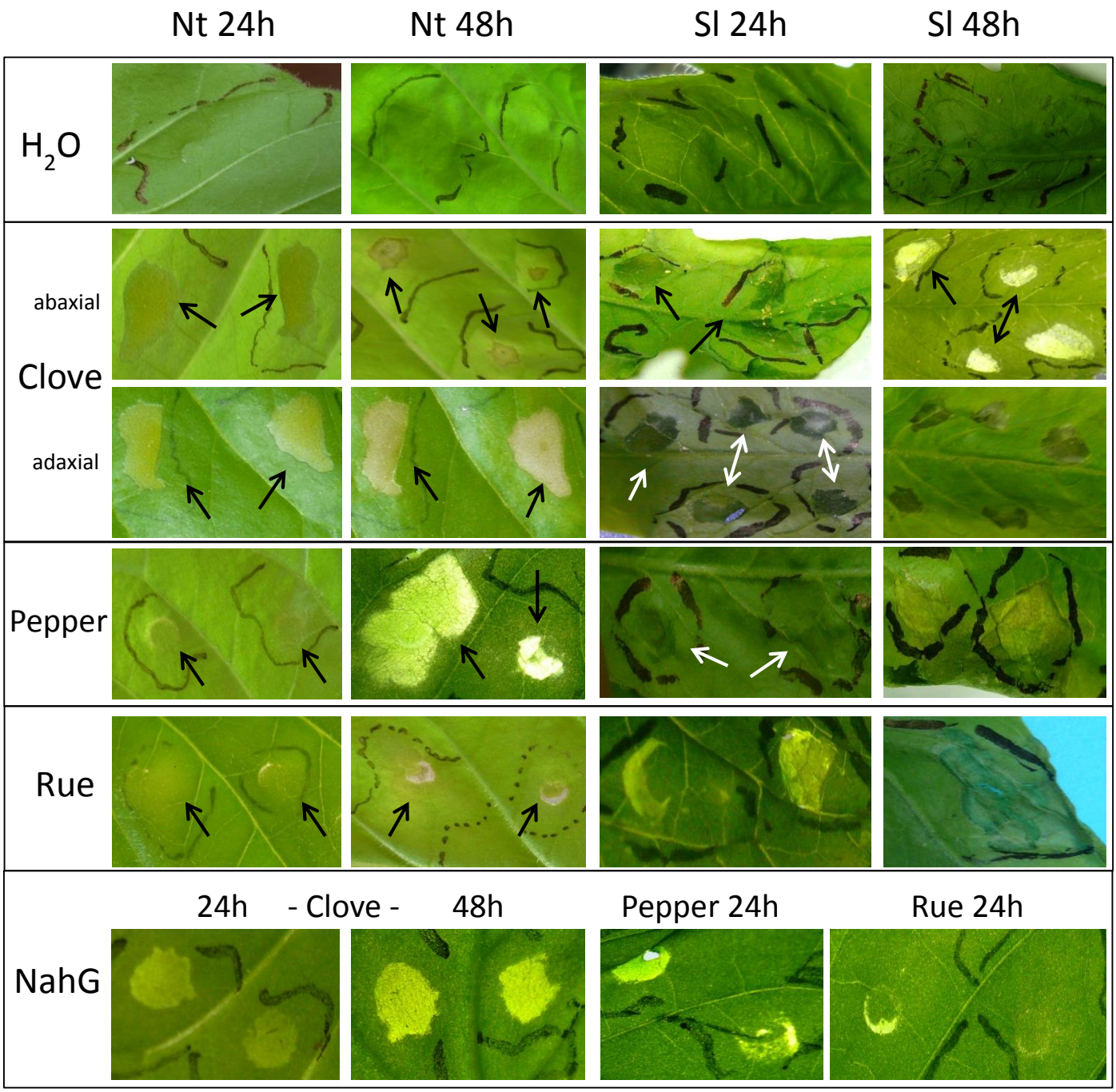
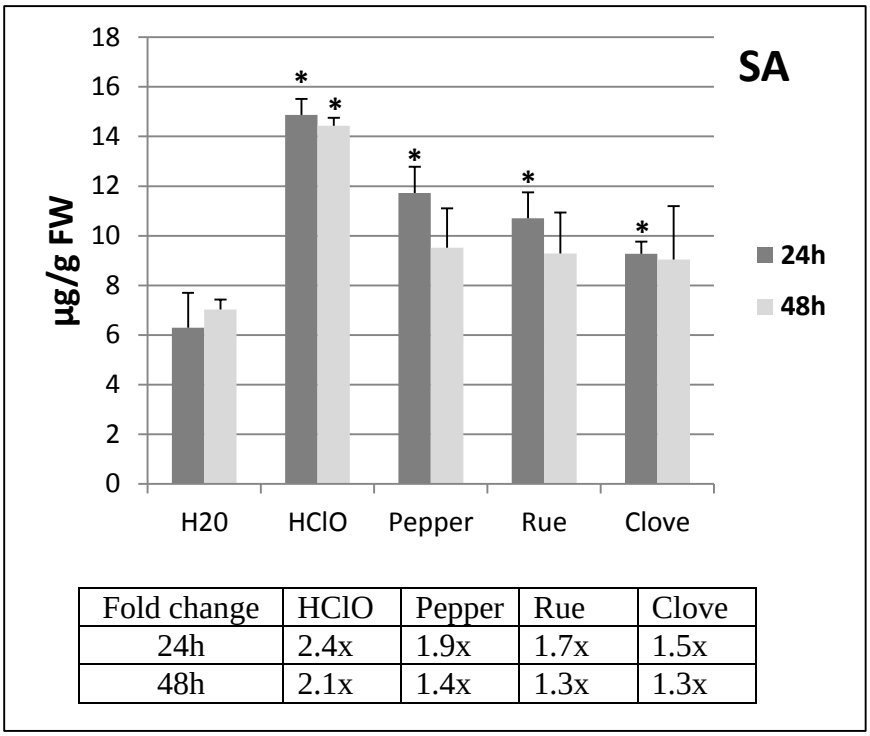


Figure 4 Macroscopic necrotic symptoms induced in WT tobacco (Nt), tomato (Sl) and transgenic NahG tobacco (NahG) leaves by pepper, wild rue and clove extracts observed 24 and 48 hours after infiltration. Negative control (H_2O) is shown. Distinct areas of necrosis are indicated by arrows.



Fold change	HClO	Pepper	Rue	Clove
24h	2.4x	1.9x	1.7x	1.5x
48h	2.1x	1.4x	1.3x	1.3x

Figure 5 Salicylic acid levels in tomato leaves 24 and 48 hours after treatment with different plant extracts (pepper, wild rue and clove) expressed as µg/g fresh weight (FW). Active chlorine (HClO) was used as positive control. The table shows the SA amount expressed as fold change compared to the negative control treated with water. Averages +SD, n = 3. The star (*) denotes a value significantly different from the negative control by ANOVA test.

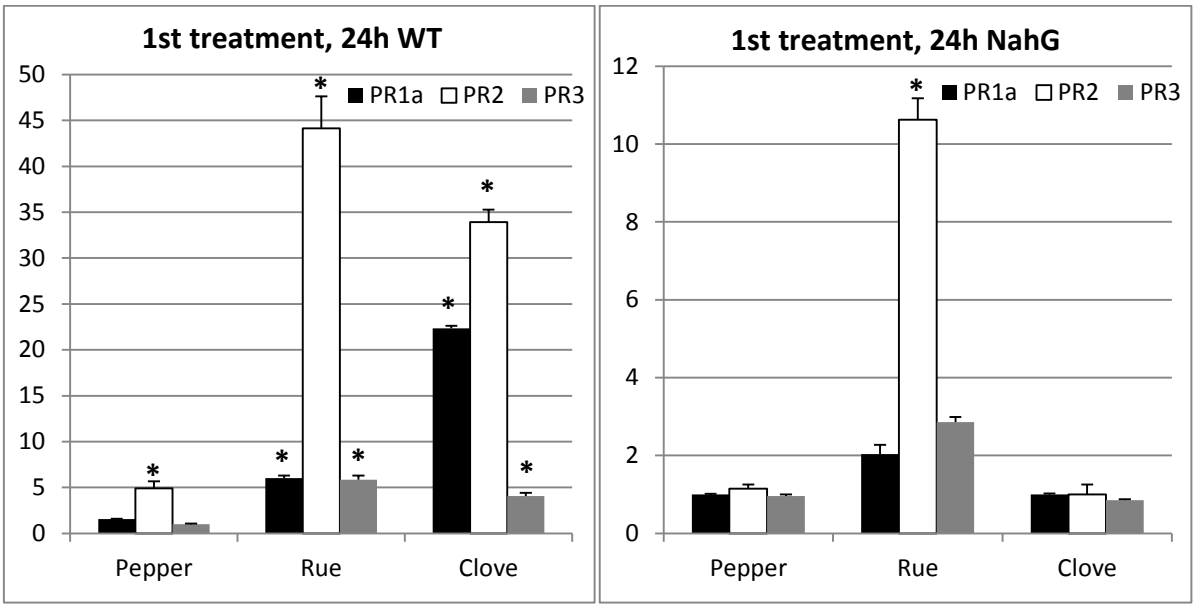


Figure 6 Levels of gene induction (fold change) of different defense genes (PR1, PR2, PR3) in WT and NahG transgenic tobacco leaves 24 hours after treatment with different plant extracts: pepper, wild rue and clove. The expression level obtained by RT-qPCR was compared to the mRNA level in negative control plants (treated with water) using the GeNorm algorithm. Average fold change +SD, n = 3. The star (*) denotes a value significantly different from the negative control (water) by t-Student test.