Ecotoxicology (2015) 24:1996–2008
DOI 10.1007/s10646-015-1535-4

The use of ECAS in plant protection: a green and efficient antimicrobial approach that primes selected defense genes

Marco Zarattini[1] · Morena De Bastiani[1] · Giovanni Bernacchia[1,2] · Sergio Ferro[1] · Achille De Battisti[1]

Accepted: 31 August 2015 / Published online: 9 September 2015

Abstract The use of highly polluting chemicals for plant and crop protection is one of the components of the negative environmental impact of agricultural activities. In the present paper, an environmentally friendly alternative to pesticide application has been studied, based on the so-called electrochemically activated solutions (ECAS). Experiments have been carried out, by applying ECAS having different contents of active ingredients, on tobacco plants at a laboratory scale and on apple trees at fruit garden scale. The results, accumulated during a couple of years, have shown that properly selected dilute solutions of chlorides, once activated by an electrochemical treatment, exhibit a very effective protecting action of plants, irrespective of their nature. Extension of the research has shown that the observed effect is the result of two distinct factors: the expected anti-microbial action of the electrochemically synthesized oxidants, and an unexpected priming of immune plant defenses, which is clearly due to the treatment with ECAS. Interestingly, the repetition of ECAS application triggers an even stronger activation of defense genes. No oxidative damages, due to the use of the activated solutions, could be detected.

Keywords Electrochemically activated solutions · Endogenous plant defense · Antimicrobial activity · Green chemicals · Oxidative stress · Hypochlorous acid

Introduction

Since infestation by pests and diseases can severely reduce the yield of agricultural and horticultural crops, one of the main concerns of modern agriculture is the fight against pathogens. In order to protect crops, before and after harvest, chemical products (generically and collectively referred to as 'pesticides') are typically used. In some cases, these chemicals act by confusing insects or making crops less palatable for pests; more commonly, the effectiveness is directly exerted by killing the undesired insect, fungi or bacteria. Obviously, such pesticides could have severe undesirable effects if they are not strictly regulated. Since the entry into force, in 2009, of European Directive 2009/128/EC (which explicitly refers to the sustainable use of pesticides), no plant protection product can be used within the Member States, unless it has first been scientifically established that:

(1) it has no harmful effects on consumers, farmers and local residents and passers-by;
(2) it does not cause unacceptable effects on the environment;
(3) it is sufficiently effective against one or more pests.

Based on the above, the use of environmental- and human-friendly molecules has become increasingly important, especially in the last few years. Researching for active substances capable of killing pathogens without harming the environment, the so-called electrochemically activated solutions (ECAS) captured our attention. ECAS

Marco Zarattini and Morena De Bastiani have contributed equally to this study.

✉ Giovanni Bernacchia
bhg@unife.it

✉ Sergio Ferro
fre@unife.it

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy

[2] Present Address: Becario Prometeo, Universidad Politécnica Salesiana, Calle Vieja 12-30 y Elia Liut, Casilla 2074, Cuenca, Ecuador

are slightly saline solutions that, after electrolysis, contain reactive oxidative species (ROS) characterized by a short life span and a potent antimicrobial activity (Hyang et al. 2008 and references therein; Thorn et al. 2012; Mukhopadhyay and Ramaswamy 2012). The electrochemical treatment of a halogenides-containing solution typically leads to the formation of hypohalogenides (e.g., hypochlorite), which are well known not only because they are effective disinfectants (FAO and WHO 2009; Gómez-López 2012), but also for their capability in removing stains and odors, as well as for their bleaching properties (A.I.S.E. scientific dossier 1997). Actually, hypochlorous acid (HOCl) is also an important component of vertebrate nonspecific immune system. Thanks to the action of the myeloperoxidase enzyme, it is synthesized from hydrogen peroxide (H_2O_2) and chloride anion (Cl^-), during the neutrophils' respiratory burst (Klebanoff 2005).

On the other hand, plants have developed complex strategies of defense. Differently from animal immune system, plants do not have a mobile immune system but each cell is able to trigger a defense mechanism. These defenses can be divided into two classes: passive and active (Hammond-Kosack and Parker 2003; Dangl and Jones 2001; Niks and Marcel 2009; Ahmad et al. 2010). Passive or constitutive defenses are physical or chemical barriers, always present in plant cells and tissues. These basic defenses can be easily overcome by the pathogen, especially if it manages to enter plant tissues through wounds or by enzymatic action. Yet, the plant can still fight back the pathogen action, by active or inducible defenses, deployed as a consequence of pathogen recognition by the plant. In fact, recognition is the key factor for the success of the strategies employed and it has a genetic base (Ahmad et al. 2010). This recognition activates a more robust multilevel defense (Jones and Dangl 2006; Bent and Mackey 2007; Carr et al. 2010). The general pattern of this active resistance is based on three important levels (Jones and Dangl 2006; Bernoux et al. 2011). Firstly, a rapid and powerful hypersensitive response (HR) weakens the pathogens near the site of infection through an oxidative burst (Wojtaszek 1997; Gozzo 2003).

Besides, from the infection site, a series of molecular signals are sent to the whole plant (ROS, salicylic acid or jasmonic acid, azelaic acid (Conrath 2011; Conrath et al. 2015)) triggering a broad and potent defense in neighboring tissues. This kind of defense is very powerful and involves the synthesis and accumulation of a heterogeneous class of proteins, the so-called PR (pathogen related) proteins, which have different chemical and physical properties (Tuzun and Somanchi 2006). The latter are induced in plants resistant not only to microorganisms but also to other biotic (insects, nematodes, herbivores) or abiotic stresses (Van Loon et al. 2006). 17 classes of PR proteins are known so far (Van Loon et al. 2006; Sels et al. 2008): most of them show antimicrobial and antifungal activity (chitinases, glucanases, proteinases). These phenomena trigger a further defensive wave that extends to distal organs creating an immunity called *systemic acquired resistance* (SAR). During SAR, plant tissues are prompted to induce a strong defense response upon new infections (Ahmad et al. 2010) thanks to one or more signals (SA, JA and others) (Pieterse et al. 2009; Shah 2009; Shah et al. 2014) that originated from the initial site of infection and spread to the whole plant (Heil and Bostock 2002). These three coordinated steps often ensure that the plant stays healthy, with no disease symptoms.

The fight against diseases in agriculture is usually based on the use of active substances toxic for the pathogen, and often also for the environment and humans. The use of molecules able to activate the endogenous plant defenses is still uncommon, even if it has been receiving a lot of attention in the last few years (Shah et al. 2014; Du et al. 2012; Bai et al. 2011; Chaturvedi et al. 2012).

A prompt from a direct field experience

In order to test a non-conventional plant-protecting treatment, in 2005 an extensive field experiment was carried out in a 2 ha apple orchard severely infected by *Nectria galligena*. The situation was so serious that agronomists contacted to resolve the problem could but suggest uprooting of orchard trees and wood incineration. At that time, our experiences with ECAS were just at the beginning, and we thought to spray the trees with the disinfectant solution, as suggested in initial papers on the subject (e.g., Al-Haq et al. 2005; Buck et al. 2003, and literature therein). Quite surprisingly, the treatment caused a drastic interruption of the pathogenic cycle and a robust healing of the lesions. At the same time, plant growth and crop production were considerably improved. As a possible comparison (control), untreated neighboring orchards of the same nature and with the same infections remained affected by the diseases and have been explanted 1 year later.

To understand these phenomena, we started an investigation based on molecular and cellular analyses, in order to understand whether the ECAS treatments were only responsible for a superficial sanitation of the infected plants or they were also able to induce specific plant defense strategies. The study took into consideration both *Nicotiana tabacum* (tobacco) and *Malus domestica* (apple) plant species. The former is often considered as a model in plant biology, while the latter is interesting especially for its commercial value.

Here we report results suggesting that ECAS treatments are able to trigger endogenous plant defenses, in particular genes belonging to the PR family and other cellular

defenses. Accordingly, the ECAS seem to represent a new-generation of active substances that combines a powerful antibacterial and antimicrobial effect, a marked effect on the plant immune system and the absence of environmental impact.

Materials and methods

Preparation of electrochemically activated solutions

As briefly discussed in a recent review by Reynolds and coworkers (Thorn et al. 2012), electrochemically activated solutions can be synthesized with different approaches. Investigations began in Russia in the early 70 s, thanks to the work of Academician V. Bakhir (Bakhir 1985; Prilutsky and Bakhir 1997), who devised a flow-through electrochemical module, based on concentric electrodes separated by a ceramic membrane (Bakhir et al. 1995). A separator between the electrode compartments is required when an *anolyte* (an oxidizing, generally acidic solution) and a *catholyte* (a reducing, alkaline solution) are sought. In addition, a neutral anolyte can be produced by suitably adjusting the (hydraulic) mixing of the above liquids. In the presence of chlorides, gaseous chlorine (Cl_2) is synthesized at the anode of the electrochemical reactor. Once formed, chlorine dissolves in water producing a *free* (or *active*) *chlorine* solution. The solution pH decides the forms in which chlorine is present, hypochlorous acid (HOCl), hypochlorite (ClO^-) or gaseous chlorine (Cl_2), and eventually represents the key factor to explain the bactericidal effectiveness of the anolyte (Len et al. 2000; Abadias et al. 2008; Xiong et al. 2010).

In the present investigation, we took into account two specific pH values (6.5 and 9.0), in order to assess the roles of hypochlorous acid and hypochlorite ion (these two species are related by a chemical equilibrium: $HOCl + H_2O \Leftrightarrow ClO^- + H_3O^+$, pKa $\approx$ 7.53). At the lower pH, most of the *active chlorine* is present as HOCl (90 %), the hypochlorite form contributing for the remaining 10 %; at pH 9, on the contrary, the opposite is observed (HOCl 5 %, ClO^- 95 %).

Solutions were obtained by starting from dilute brines (5 g L^{-1} of either NaCl or KCl, in tap water), which were electrolyzed for a few minutes in electrochemical reactors provided with or without a separator, in order to obtain the desired pH values. After the synthesis, the solution pH was measured with a pH-meter and adjusted to the required value by adding small amounts of hydrochloric acid or sodium hydroxide. The *active chlorine* content (always comprised between 700 and 1000 mg L^{-1}) was quantified by means of the standard *N,N*-diethyl-1,4-phenylenediamine colorimetric method, on specimens diluted with distilled water (ISO 7393-2 2002).

Plant materials

Plants of *Nicotiana tabacum* cv. Petite Havana SR-1 and *Malus domestica* cv. Fuji were grown in a controlled growth chamber with a 16/8 h photoperiod (150 µE m^{-1} s^{-1}) at 24 °C and 60 % of humidity. The mutant tobacco plants *NahG* (Friedrich et al. 1995) (kindly provided by Dr. Luis Mur, University of Aberystwyth, Wales, UK), unable to accumulate salicylic acid, and the transgenic tobacco plants carrying the GUS reporter gene under the control of the pathogen related gene PR1 (*PR1a-GUS* (Grüner and Pfitzner 1994), kindly provided by Dr. Ursula Pfitzner, University of Hohenheim, Stuttgart, Germany) were grown in the same conditions. Field tests were done in a local farm on a parcel of *Malus domestica* cv. Dallago (20 years old).

Treatments and samplings

Electrochemically activated solutions containing 250 mg L^{-1} of *active chlorine* (*AC*) and adjusted at the desired pH (6.5 or 9.0) were sprayed on plants of tobacco (2-months old) and apple (1-year old), until their complete wetting. In parallel, plants of tobacco and apple were sprayed with a control non-electrolyzed solution, having a similar pH and chloride content. 2, 6, 18, and 24 h after the first treatment with ECAS and control solutions, the fourth and fifth leaves were harvested; upon a second treatment, leaf samples were collected after 2, 6, 12, 24, 48 or 96 h.

In field tests, due to the hardiness of trees, the concentration of ECAS and control solutions was increased to 500 mg L^{-1} and leaf pool were collected at 24, 48, and 96 h.

After sampling, the leaves were immediately frozen in liquid nitrogen, grounded in liquid nitrogen and stored at −80 °C.

RNA extraction and real-time PCR

Total RNA was extracted from 100 mg of powdered leaves by "Spectrum™ Plant Total RNA Kit" (Sigma). To avoid genomic DNA contaminations, a DNAse treatment was carried out as suggested by the manufacturer. Total RNA integrity was then controlled on 1 % (w/v) agarose gel, while the genomic contamination was assessed by conventional PCR. The relative quantification of mRNAs was performed by means of a two steps real-time analysis. In the first step, single-strand cDNA was prepared using iSCRIPT™ cDNA synthesis Kit (Bio-Rad). In the second step, the real-time PCR was performed using a SYBR

green I mix (SsoFast EvaGreen Supermix-Bio-Rad) on a Chromo4 real-time PCR system (Bio-Rad). The efficiency of the cDNA synthesis was evaluated by real-time PCR amplification using the control Actin gene. Only the cDNAs that showed an Actin Ct value of 22 ± 2 were considered for the subsequent gene expression analyses. Chosen genes are listed in Table 1.

The relative expression level of all genes investigate were obtained using the geNorm algorithm (geNorm 2008). This algorithm requires the use of two stable reference genes (in our case Actin and Elongation Factor 1α). At each time, the treated and control samples were compared with geNorm. qPCR reactions have been performed in triplicate and intra-assay variance was considered acceptable when the standard deviation was lower than 0.5. Given the high biological variability, another specific algorithm has been used to compare the replicates (Vandesompele et al. 2002; Willems et al. 2008).

The results obtained have been expressed as "fold changes", *i.e.* as the over-expression of the genes induced by the electrolyzed solution, compared to the control solution.

Histochemical analyses

Superoxide and H_2O_2 staining

The $O_2^{\bullet -}$ and H_2O_2 production was evaluated by histochemical staining with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively. After ECAS treatment, full leaves from treated and control plants were cut at different time points and the in situ presence of superoxide and hydrogen peroxide was evaluated as described previously (Jabs et al. 1996; Dutilleul et al. 2003; Thordal-Christensen et al. 1997). Positive control plants were treated with Paraquat 20 μM.

GUS histochemical assay

To evaluate if the treatments with ECAS were able to induce the SAR molecular marker PR1a, transgenic plant PR1a-GUS were treated either with ECAS or with the control solution. The GUS histochemical assay was carried out as previously described (Vitha et al. 1995). Briefly, tobacco leaves harvested 6 and 24 h after the treatment were incubated for 30 min in 5 mL of fixative buffer, washed several times for 30–40 min in washing buffer and vacuum-infiltrated in the X-gluc substrate for 30 min. Staining was performed in darkness at 37 °C for 16–20 h. To remove chlorophyll, leaves were washed with 70 % ethanol. Photographs were taken with a Nikon Eclipse E200 optical microscopy.

Results and discussion

Results of gene expression on tobacco

Electrochemically activated solutions are potent sterilizing solutions, able to kill several kinds of phyto-pathogenic microorganisms. Their application on vegetative parts of diseased plants may heal the plant organs but it is not clear whether this effect is only due to the biocide activity or there is also a positive effect on the plant. To evaluate the plant responses to ECAS treatment, we chose healthy tobacco plants and analyzed the expression profiles of several genes involved in plant defenses against pathogens (members of the PR family, enzymes and regulatory proteins) comparing the fold change observed in ECAS-

Table 1 Panel of analyzed genes, for both tobacco and apple plants, with respective GenBank accession numbers

Gene	Function	Tobacco	Apple
PR1a	Antifungal	X06361	DQ318212
PR2	β-1,3-glucanase	M60460	AY548364
PR3a	Acidic Chitinase	X51426	AF494395
PR4a	DNA-RNAse?	X58547	
PR5	Thaumatin-like, antifungal	S44889	DQ318213
PR8	Endochitinase		DQ318214
PR17	Unknown	AB024600	
PAL	Phenylalanine ammonia-lyase	D17467	AF494403
WRKY3	Transcriptional factor	AF193770	
RBOH	Hypersensitive response-related protein	AJ309006	
HSR203 J	Hypersensitive response-related protein	X77136	
Actin	Reference	U60493	GQ339778
EFNT1α	Reference	AF120093	DQ341381

treated plants with control-treated plants. The relative quantification of the mRNA levels for each gene was performed using real-time PCR by considering two other genes as reference (Actin and EF1α). To take into consideration the PCR efficiency, the geNorm algorithm was employed (geNorm 2008; Vandesompele et al. 2002).

Tobacco plants were treated with ECAS (250 mg L^{-1}) or control solution, and leaf samples collected at different times (2–24 h) from different plants, in order to exclude wound-induced responses. Then, total RNA was extracted, retro-transcribed and amplified in real-time PCR. PR and other defense-related genes were examined and their expression level normalized with reference genes. In ECAS-treated plants, the activation of genes was expressed as fold change in comparison with control-treated plants. The results shown in Fig. 1 reveal that a single ECAS treatment can increase the expression level of several PR genes (especially PR1a, PR2 and PR17, tenfold increase) starting from 6 h after the treatment. No PR up-regulation was detected 2 h after the ECAS treatment, while at later stages only a few genes were still expressed (PR1a, PR2, PR3a and PR17). A significant and fast up-regulation was also observed for the HR-related gene HSR203 J during the first 2–6 h, while PAL and WRKY3 genes showed a tenfold increase 6 h after treatment and then returned to basal levels. Other genes (NPR1, non-expressor of PR1, is a key regulator of systemic acquired resistance; ICS, isochorismate synthase; PR2a, basic Class I glucanase; and PR3, basic class I endochitinase) were also analyzed but did not show any induction upon treatment.

Control-treated plants did not show significant differences in terms of gene expression profiles when compared to untreated plants (data not shown), confirming that the activation observed after treatment with ECAS was not merely due to the dilute brine solution itself, but rather to the (more or less labile) chemical species generated by electrochemical activation.

The same tobacco plants were treated again with ECAS 14 days after the first treatment. This allowed determining whether plant molecular defense responses vary with time and if there was a priming phenomenon associated with ECAS treatments. Leaf samples were collected at different time points from independent plants (to avoid wound-induced effects) and tested in quantitative real-time. Figure 2 reveals that a second ECAS treatment enhances PR gene expression, especially 24 h after the treatment, with very high levels of induction for PR1a and PR2 (up to 1000× fold change). Other PR genes showed expression profiles similar to those observed after the first treatment. In terms of the other genes analyzed, PAL gene was again rapidly induced at high levels (40×) but later its expression level decreased. Interestingly, most of the genes analyzed appear to be still overexpressed 48 h after the treatment; this outcome was not observed after the first treatment. No sign of phyto-toxicity was ever observed.

When the second treatment was performed on tobacco plants 35 days after the first treatment, the gene expression analysis revealed a significant activation of several PR genes (PR1a 100× fold change) that lasted up to 96 h. On the other hand, there were no differences in the expression levels of other defense genes (PAL, WRKY3, etc.; data not shown). This would suggest that ECAS treatment can induce a priming phenomenon that appears quite strong during the first weeks and then it decreases in intensity while remaining still detectable after 1 month.

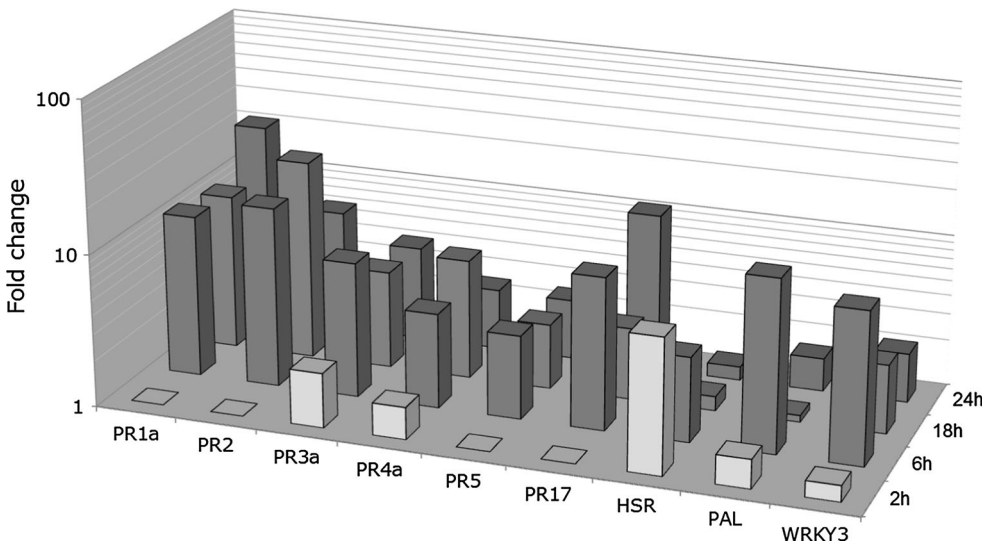

Fig. 1 Gene expression on tobacco plants, treated once with ECAS containing 250 mg L^{-1} of *AC* at pH 6.5. Fold-change values presented a standard deviation always lower than 20 %

Dependence of gene expression on ECAS concentration and pH

To establish if the observed gene induction is influenced by the concentration of active chlorine present in the electrochemically activated solution, we sprayed tobacco plants also with solutions containing 125 and 500 mg L^{-1} of *AC*. Leaves were collected from independent plants after 12 and 48 h. After a first treatment, quantitative real-time amplification and fold change analysis revealed that the induction in the expression of the defense-related genes (both PRs and other genes) was comparable to those of the previous treatments (with 250 mg L^{-1} of *AC*, data not shown). On the other hand, a second treatment 14 days after the first revealed that the priming phenomenon is much more intense with ECAS at 250 mg L^{-1}. At 125 mg L^{-1}, the defense-genes overexpression was quite weak, even weaker at 500 mg L^{-1} as compared to the overexpression observed with ECAS at 250 mg L^{-1} (data not shown).

These molecular data thus suggest that an *AC* content of 250 mg L^{-1} is an optimal concentration for tobacco plants since it allows achieving the highest expression level for the longest period. It is worth noting that no symptoms of phyto-toxicity were observed on plants treated even at higher ECAS concentrations (up to 800 mg L^{-1}, data not shown).

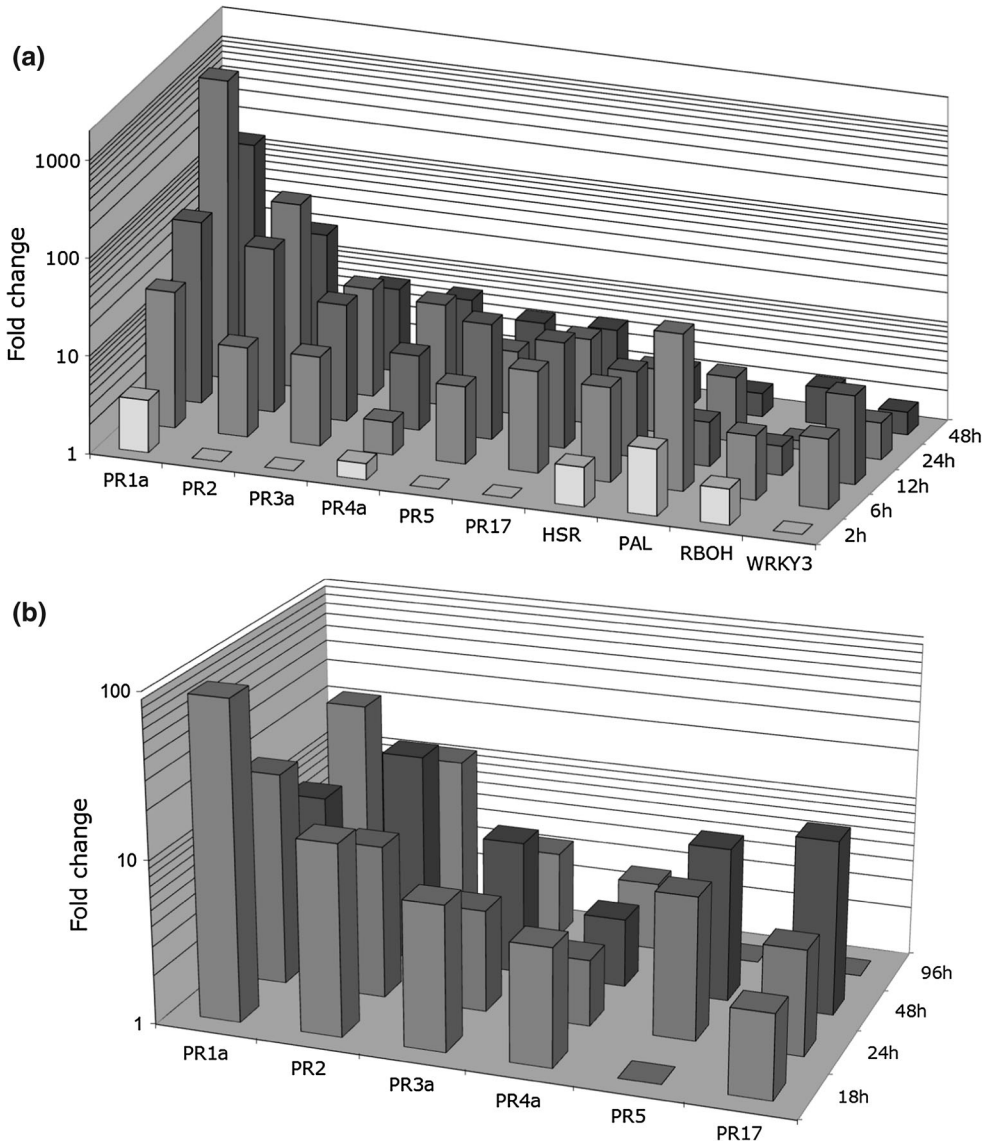

Fig. 2 Gene expression on tobacco plants, treated twice with ECAS containing 250 mg L^{-1} of *AC* at pH 6.5. **a** The 2nd treatment was performed 14 days after the first one; **b** the 2nd treatment was performed 35 days after the first one. Fold-change values presented a standard deviation always lower than 20 %

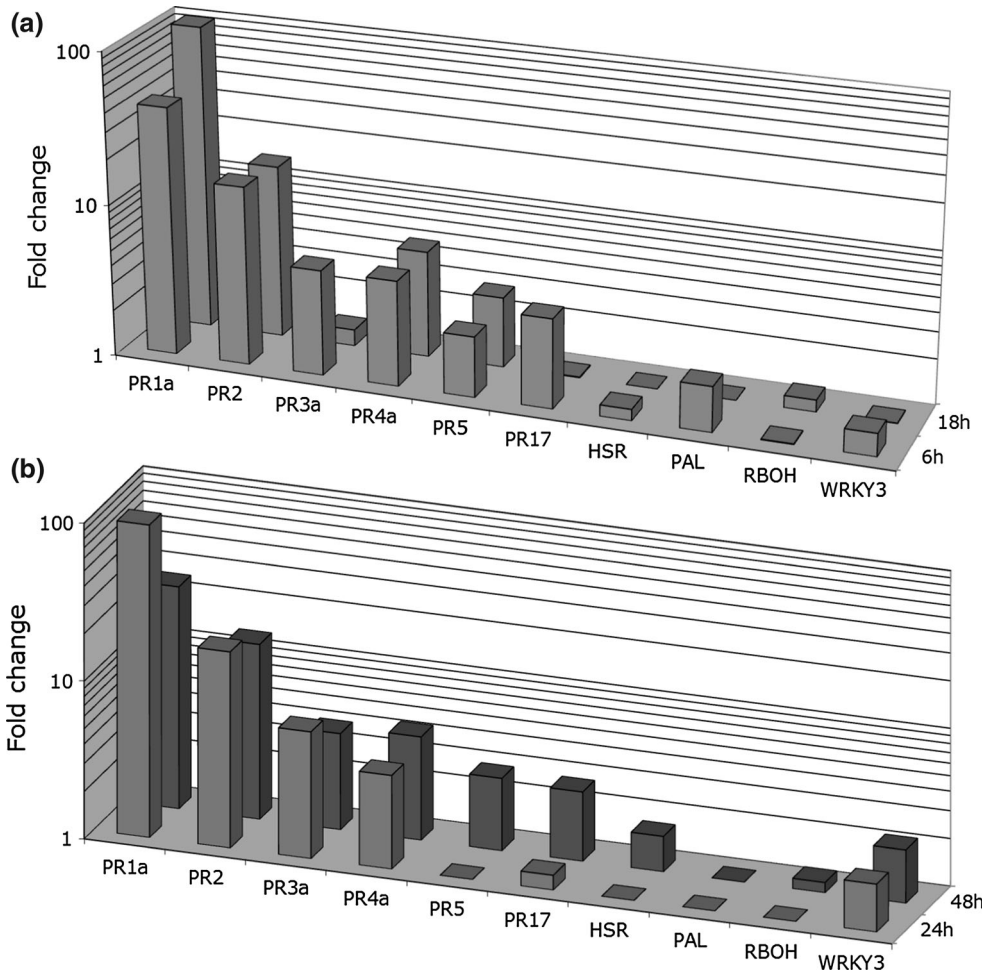

Fig. 3 Gene expression on tobacco plants, treated with ECAS containing 250 mg L^{-1} of *AC* at pH 9.0. **a** 1st treatment; **b** the 2nd treatment was performed 14 days after the first one. Fold-change values presented a standard deviation always lower than 20 %

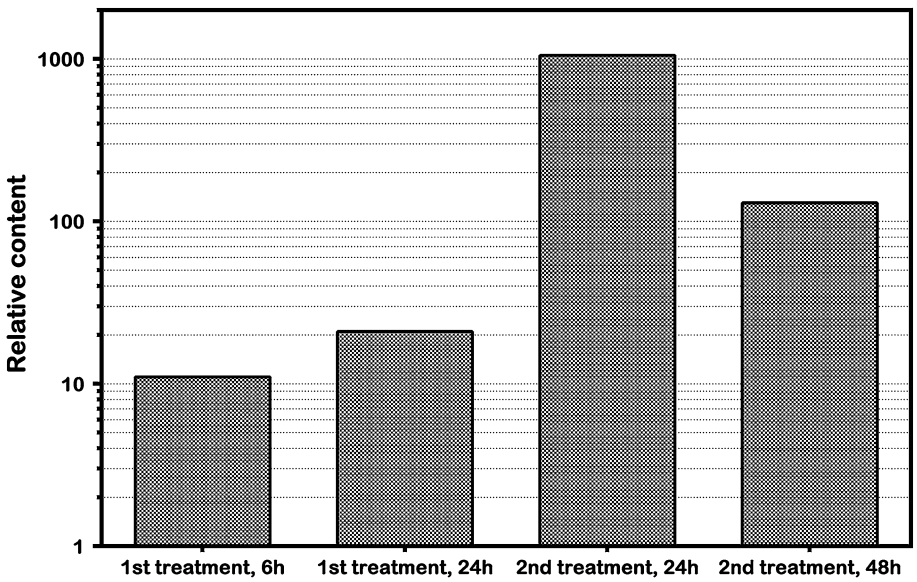

Fig. 4 Salicylic acid content in tobacco leaves sprayed with ECAS containing 250 mg L^{-1} of *AC*, at pH 6.5. Data refer to the first and second treatments (after 14 days)

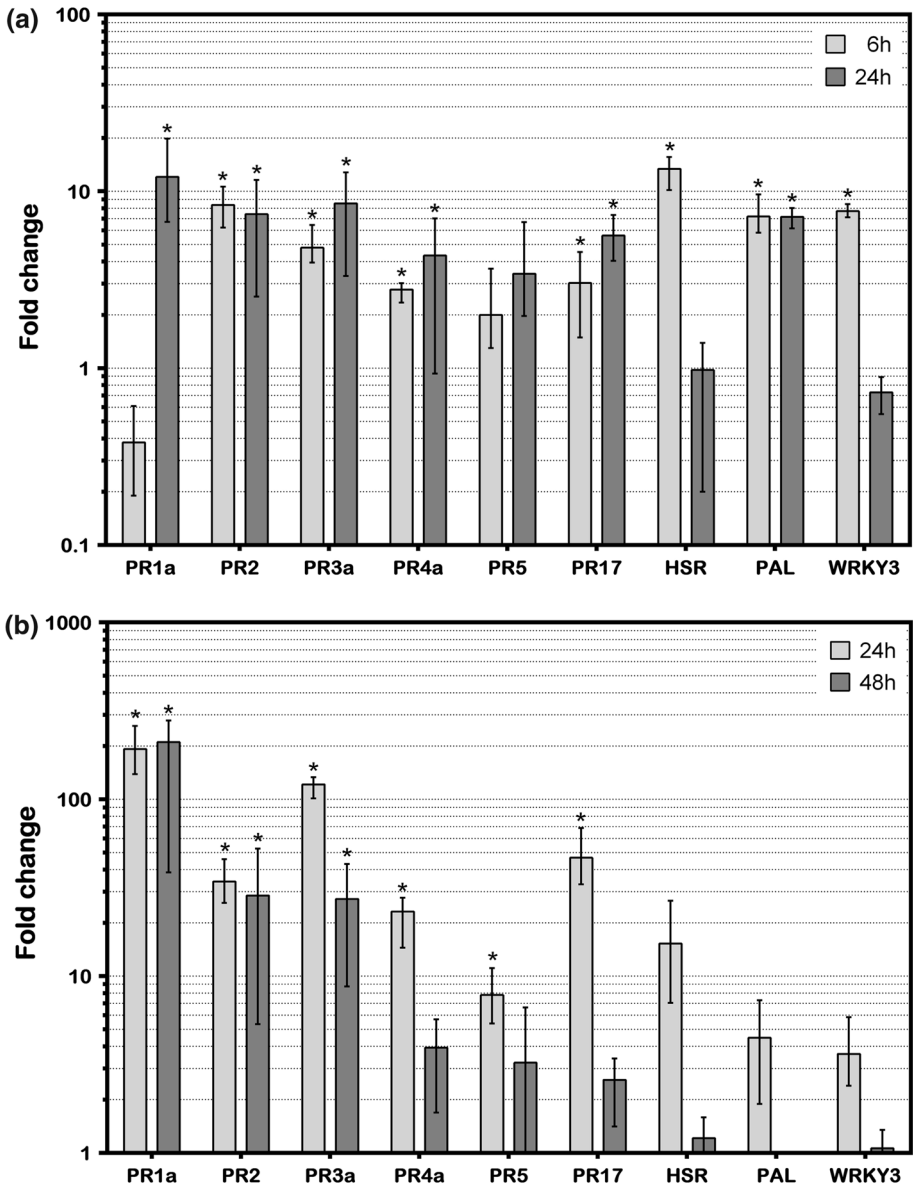

Fig. 5 Gene expression on transgenic (*NahG*) tobacco plants, treated with ECAS containing 250 mg L^{-1} of *AC* at pH 6.5: **a** 1st treatment; **b** 2nd treatment (after 14 days). *Stars* indicate data for which a statistical significance has been ascertained according to Student's *t* test ($p < 0.05$)

We also assessed if the observed gene overexpression was linked to the pH value of the electrochemically activated solution. As previously mentioned, the pH value of the solution changes considerably the nature of the molecules present in the electrolyzed solution. At neutral pH, hypochlorous acid is prevalent, while at alkaline pH the hypochlorite form is predominant. We treated tobacco plants with ECAS containing 250 mg L^{-1} of *AC* at either pH 6.5 or pH 9.0, and carried out mRNA analyses via real-time PCR on leaves collected at various time points. As shown in Fig. 3, the electrochemically activated solution with alkaline pH triggers an overexpression that is limited to some of the PR genes (PR1a and PR2), while other genes (PRs, PAL, HSR, RBOH and WRKY3) are not up-regulated. When a second treatment is performed 14 days after the first, a partial priming and enhancement of gene expression has been evidenced by real-time PCR analysis 24–48 h after the treatment, but they are not comparable to the enhancement obtained with electrochemically activated solutions at pH 6.5. PR1a and PR2 show a 100× and a 10× fold increase, respectively, with ECAS at pH 9 (24 h) while they showed a 1000× and a 100× fold increase 24 h after the second treatment in the case of ECAS at pH 6.5. The other defense-related genes were only slightly over-expressed, while with ECAS at pH 6.5 they were significantly over-expressed in both the 24 and the 48 h samples.

Accordingly, these analyses indicate that hypochlorous acid, rather than hypochlorite, is essential to achieve a strong and long lasting activation of plant defenses.

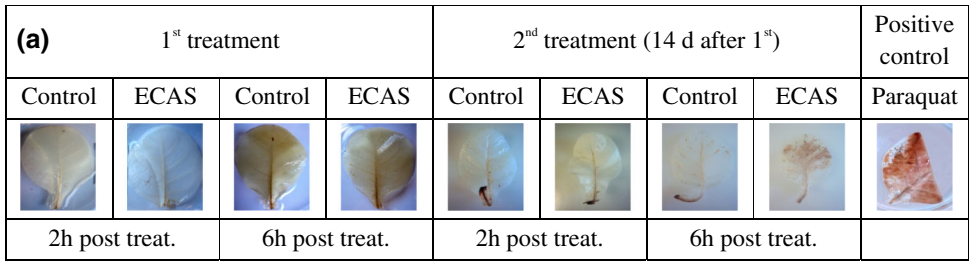

Fig. 6 DAB and NBT histochemical essays performed on tobacco leaves sprayed with ECAS containing 250 mg L^{-1} of *AC*, at pH 6.5: **a** a *brown* staining would reveal the presence of H_2O_2; **b** a *blue* staining would reveal the presence of $O_2^{\bullet -}$ (Color figure online)

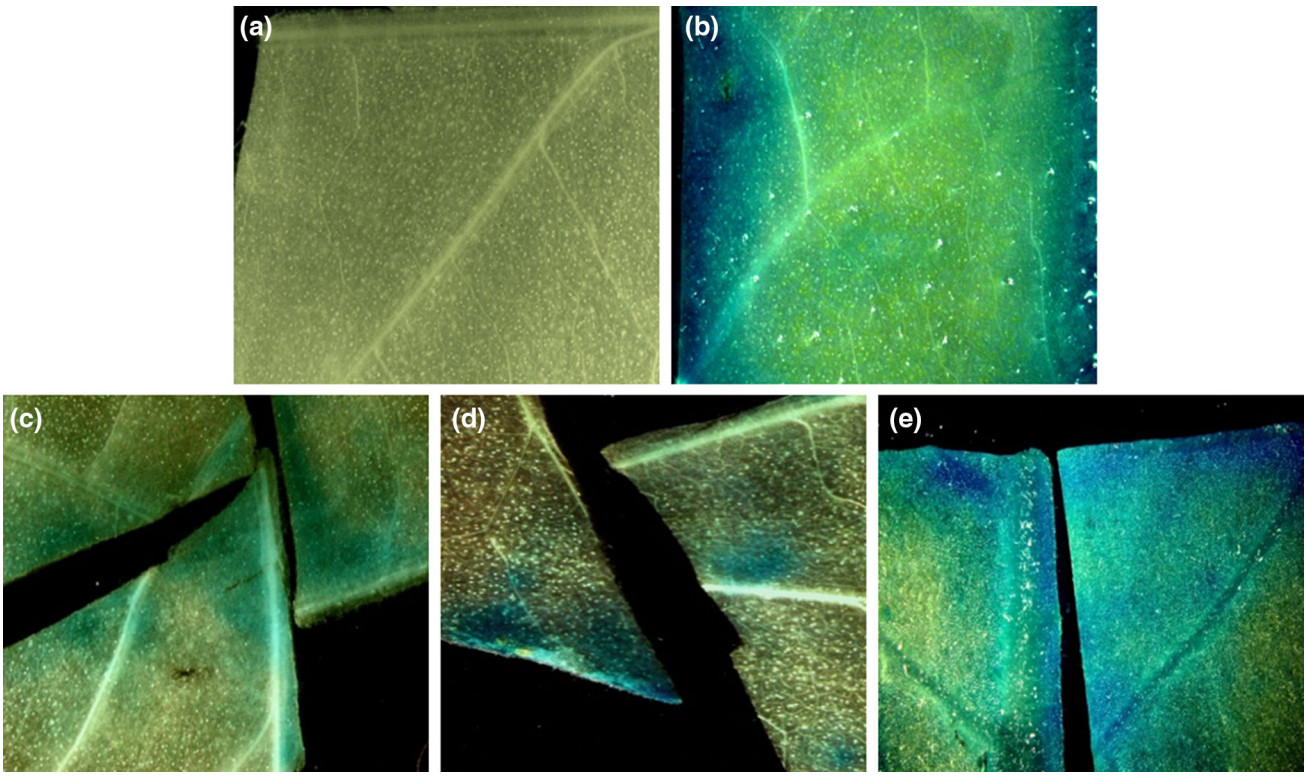

Fig. 7 GUS histochemical staining on PR1a-GUS transgenic tobacco plant after different treatments: **a** control solution, 24 h after 1st treatment; **b** positive control BTH (130 mg L^{-1}) 24 h after 1st treatment; **c** ECAS (250 mg L^{-1} of *AC*) 24 h after 1st treatment; **d** ECAS (250 mg L^{-1} of *AC*) 24 h after 2nd treatment, 14 days after the 1st; **e** ECAS (250 mg L^{-1} of *AC*) 48 h after 2nd treatment, 14 days after the 1st

Since the observed results are related to defensive, salicylic acid-dependent protein activation, it might be that ECAS is able to trigger an increase in the production of salicylic acid. The quantification of the salicylic acid hormone (an important signaling molecule, activator of the endogenous defenses) was carried out on tobacco leaf tissues collected at different time points, after a first or a second treatment (14 days). The quantification of salicylic acid in leaves was performed by GC–MS analysis (Deng et al. 2003); results, expressed as the ratio of concentrations found in the treated plants against those used as controls, are shown in Fig. 4. The chemical data confirmed those

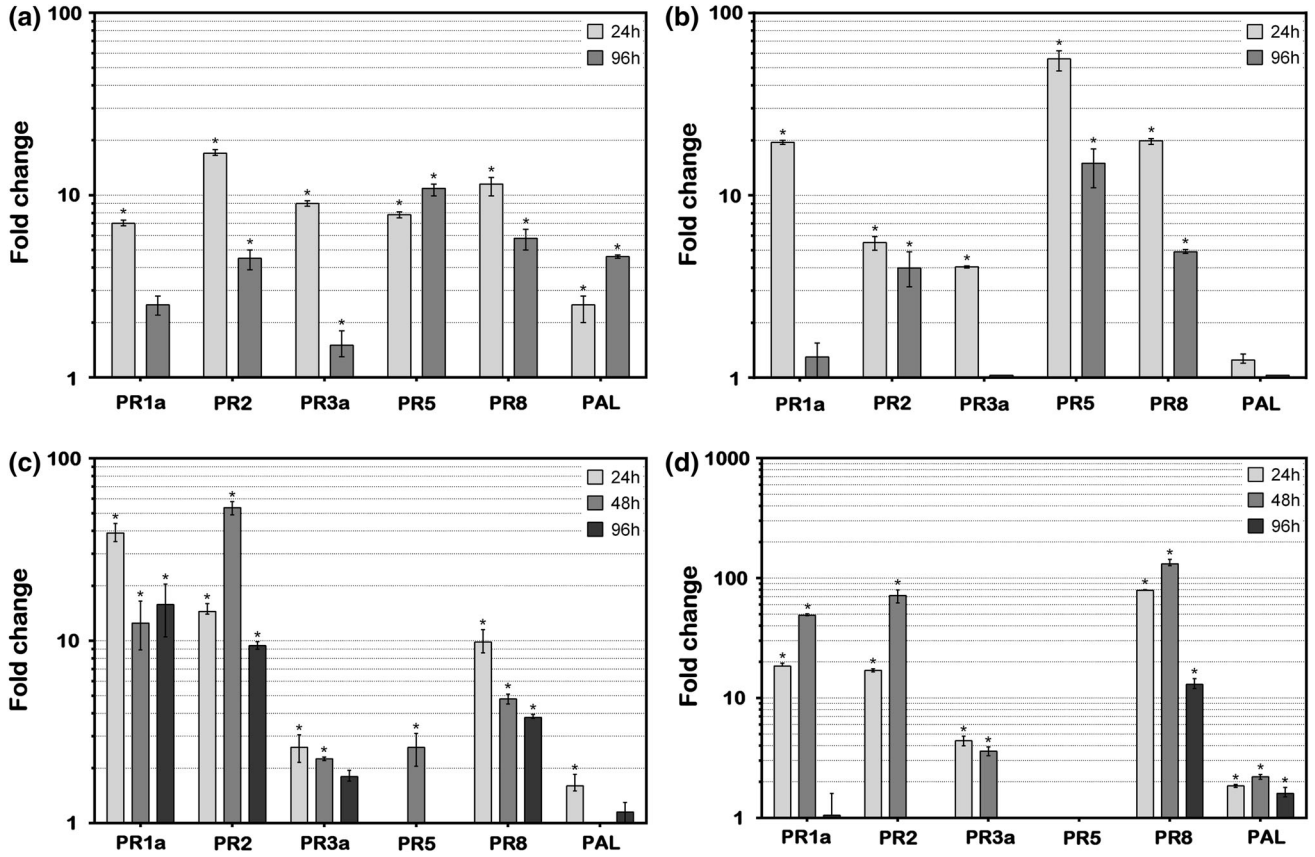

Fig. 8 Gene expression in apple plants, treated with ECAS at pH 6.5, containing either 250 mg L^{-1} of *AC* (**a**, **b**, young plants) or 500 mg L^{-1} of *AC* (**c**, **d**, plants in orchard); **a**, **c** refer to 1st treatment, while **b**, **d** refer to 2nd application (after 14 days). *Stars* indicate data for which a statistical significance has been ascertained according to Mann and Whitney's test ($p < 0.05$)

obtained by molecular analysis, proving that the signal molecule of salicylic acid is effectively synthesized after a single ECAS treatment (ten times more abundant than in control plants). The hormone levels increased drastically even after a second treatment, with an induction profile quite similar to that described for the gene activation. As a result, these data seem to suggest that salicylic acid is at least partially implicated in the immune response of ECAS-treated plants.

In order to confirm the role of salicylic acid in the ECAS-triggered up-regulation of genes, tests were carried out also on transgenic tobacco plants that are unable to accumulate this hormone (*NahG*). As can be seen from Fig. 5, tests carried out using the *NahG* plants show a gene activation profile much less pronounced compared to wild-type plants. In particular, a weak activation of PR1a, a typical salicylic-dependent gene, is evident after a single ECAS treatment and even weaker after the second treatment (maximum fold change 100× as compared to 1000× for wild-type plants). Nevertheless, many defense genes appeared activated despite the absence of SA, thus suggesting that the activation of defenses induced by treatment with ECAS takes place along with the synthesis of salicylic acid, but the latter does not seem to be the main cause of activation of genes. SA participates in the gene activation and in priming the plants, but it is not the sole responsible, therefore other pathways might be involved.

Histochemical investigations

Since the ECAS application can possibly induce an oxidative stress, we evaluated the accumulation of reactive species such as H_2O_2 and $O_2^{\bullet -}$ in tobacco leaves sprayed with ECAS through histochemical staining. The synthesis of hydrogen peroxide was analyzed using 3,3′-diaminobenzidine (DAB) (Dutilleul et al. 2003), whereas the nitroblue tetrazolium (NBT) staining method was used to highlight the presence of the superoxide anion (Dutilleul et al. 2003). Tobacco leaves were collected and stained 2 and 6 h after ECAS application; Paraquat was used as a positive control. The degree of staining of ECAS-treated leaves was not significant (Fig. 6), as compared to the positive control, thus suggesting that ECAS treatments do not generate any oxidative stress in tobacco plants.

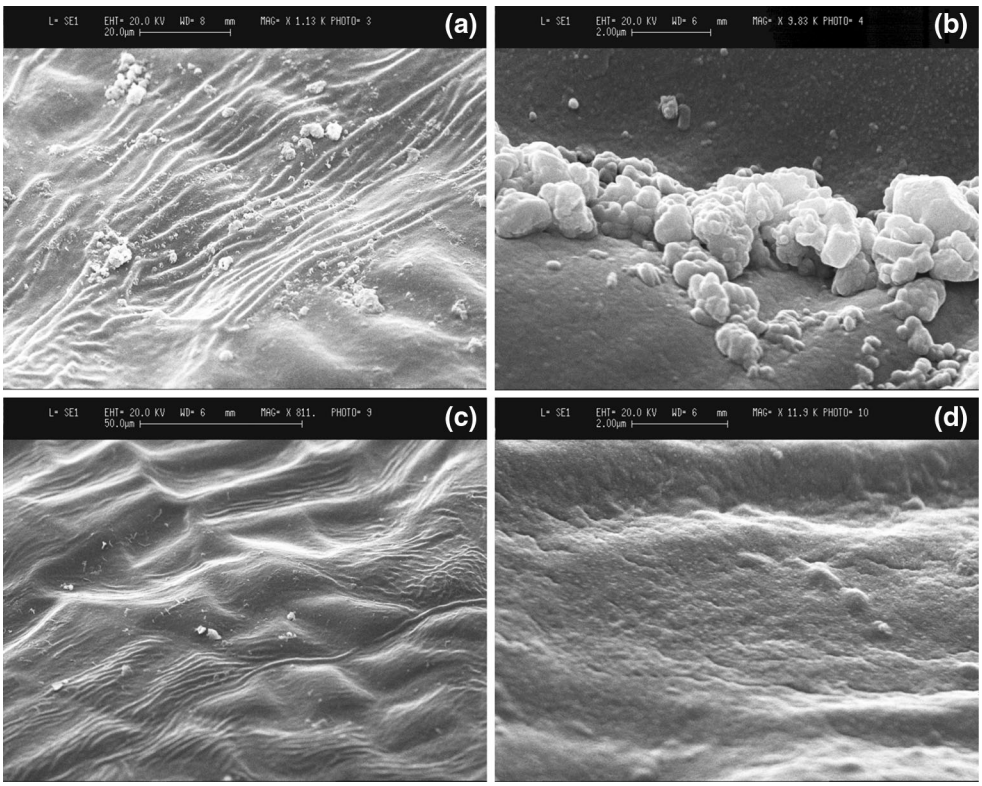

Fig. 9 SEM micrographs of apple leaves, before (**a**, **b**) and after (**c**, **d**) the treatment with ECAS at pH 6.5, containing 250 mg L^{-1} of *AC*

The gene expression data were also compared to histochemical data obtained from transgenic tobacco plants bearing a PR1a-GUS transgene able to express the GUS reporter protein as a result of the PR1a promoter activation (Grüner and Pfitzner 1994). Tobacco PR1a-GUS plants were treated with ECAS analogously to WT plants treated before and leaf samples were collected at different time points. Then, the tissues were immediately fixed and stained overnight in X-Gluc solution. After chlorophyll removal, ECAS-treated samples were clearly stained after either the first or the second treatment (Fig. 7) while control-treated plants did not show any GUS-specific staining. This evidence confirms what previously observed via real time PCR: the PR1a gene is overexpressed upon ECAS treatment and enhanced upon a delayed second treatment. As a positive control, we treated PR1-GUS plants with the SA analog BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester), a potent activator of PR1a (Görlach et al. 1996). 24 h after the treatment, an intense staining was observed due to the strong PR1a activation (also confirmed at the molecular level: PR1a 1000× fold change by RT-PCR).

Results of gene expression on Apple

Based on the molecular data obtained on tobacco, we extended the investigation and gene activation analysis to a species of great agronomic interest such as apple. ECAS applications were carried out on 1 year-old plants grown in a growth chamber, as well as on mature trees (about 20 year-old), in orchard. The young plants were treated with an electrochemically activated solution at pH 6.5 containing 250 mg L^{-1} of *active chlorine* (Fig. 8a, b). Since the old plants in the orchard showed thicker leaves and a greater vigor, as compared to those in the growth chamber, we increased the *AC* concentration to 500 mg L^{-1} (Fig. 8c, d).

Similarly to what observed in tobacco, a first treatment with ECAS is able to trigger a defensive response in apple trees. All tested PR genes as well as the PAL gene were up-regulated (10–30× fold change), especially in the case of plants in orchard, whose responses were stronger than those of plants in the growth chamber. Differently from what observed in tobacco plants, a second ECAS treatment, 14 days after the first, is capable of triggering a defensive response in apple plants, but with weak "enhancement effect". In apple trees, the gene expression remains approximately constant, with no significant variations with the gene expression level observed after the first treatment.

Organoleptic tests (color, and sugar content of apple fruits) as well as chlorophyll and total nitrogen contents (in leaves) did not indicate changes, neither in the properties and quality of fruits, nor in the vigor of treated plants (data not shown). As regards the antimicrobial effects associated

with treatments, SEM micrographs of orchard apple leaves taken before (Fig. 9a, b) and after (Fig. 9c, d) the ECAS application clearly show how the treatment substantially leads to the disappearance of any microorganism from the surfaces. Pictures taken on leaves treated with the control solution (KCl 5 g L^{-1}, pH 6.5) did not show any noticeable change as compared to untreated leaves (data not shown). This is a somewhat expected outcome: the leaves are normally subjected to bad weather, in nature, and the simple washing with water or with a diluted salt solution is not sufficient to wash away the microorganisms. On the contrary, the washing with ECAS is effective, plausibly because the active chlorine is able to destroy the biofilm (Ozaki et al. 2012) and the microorganisms' adhesion means as well. Overall, this represents a further proof of the potential of ECAS as a green alternative to other pesticides.

Conclusions

The presented results suggest that electrochemically activated solutions, thanks to their proven antimicrobial- and antifungal-activity along with the efficacy in activating the molecular defenses of plants (especially after repeated treatments) and the low phyto-toxicity, are a promising alternative to conventional pesticides. Owing to its various possibilities of attack, the active ingredient is not expected to induce resistance in the target pathogens; actually, the lack of specificity represents also the weak point of the application, which requires to be suitably timed, in accordance with plant growth and, hopefully, within an integrated pest management program. This green alternative to the pesticides has the advantage of being safe for the users and do not leave residuals on treated fruits. Lastly, but no less important, ECAS do not pose environmental risks and can be produced when required, at low costs.

Acknowledgments The authors thank Dott. Gino Masina for helpful discussion. This work was funded by ParcAgri (Ferrara, Italy) and Industrie De Nora (Milan, Italy).

Author Contributions The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Abadias M, Usall J, Oliveir M, Alegre I, Viñas I (2008) Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally processed vegetables. Int J Food Microbiol 123:151–158

Ahmad S, Gordon-Weeks R, Pickett J, Ton J (2010) Natural variation in priming of basal resistance: from evolutionary origin to agricultural exploitation. Mol Plant Pathol 11:817–827

A.I.S.E. scientific dossier (1997) Benefits and safety aspects of hypochlorite formulated in domestic product. http://aise.eu/www-old/PDF/intr_scientific.pdf

Al-Haq MI, Sugiyama J, Isobe S (2005) Applications of electrolyzed water in agriculture & food industries. Food Sci Technol Res 11:135–150

Bai W, Chern M, Ruan D, Canlas PE, Sze-To WH, Ronald PC (2011) Enhanced disease resistance and hypersensitivity to BTH by introduction of an NH1/OsNPR1 paralog. Plant Biotechnol J 9:205–215

Bakhir VM (1985) Regulating physical and chemical properties of technological aqueous solutions by unipolar electrochemical exposure and experience of its practical application. A thesis of a Dr. Science Technology—Kazan, Kazan Institute of Chemical Technologies

Bakhir VM, Zadorozhny JG, Rakhmanin JA, Naida IN, Naida NN, Dzheiranishvili NV, Leonov BI, Butin SK, Vedenkov VG inventors (1995) Apparatus for electrochemical treatment of water. United States Patent no. US 5,427,667

Bent AF, Mackey D (2007) Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. Annu Rev Phytopathol 45:399–436

Bernoux M, Ellis JG, Dodds PN (2011) New insights in plant immunity signaling activation. Curr Opin Plant Biol 14:512–518

Buck JW, van Iersel MW, Oetting RD, Hung Y-C (2003) Evaluation of acidic electrolyzed water for phytotoxic symptoms on foliage and flowers of bedding plants. Crop Prot 22:73–77

Carr JP, Lewsey MG, Palukaitis P (2010) Signaling in induced resistance. Adv Virus Res 76:57–121

Chaturvedi R, Venables B, Petros RA, Nalam V, Li M, Wang X, Takemoto LJ, Shah J (2012) An abietane diterpenoid is a potent activator of systemic acquired resistance. Plant J 71:161–172

Conrath U (2011) Molecular aspects of defence priming. Trends Plant Sci 16:524–531

Conrath U, Beckers GJM, Langenbach CJG, Jaskiewicz MR (2015) Priming for enhanced defense. Annu Rev Phytopathol 53:97–119

Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826–833

Deng C, Zhang X, Zhang J, Qian J, Zhu W (2003) Rapid determination of salicylic acid in plant materials by gas chromatography–mass spectrometry. Chromatographia 58:225–229

Du Q, Zhu W, Zhao Z, Qian X, Xu Y (2012) Novel benzo-1,2,3-thiadiazole-7-carboxylate derivatives as plant activators and the development of their agricultural applications. J Agric Food Chem 60:346–353

Dutilleul C, Garmier M, Noctor G, Mathieu C, Chétrit P, Foyer CH, de Paepe R (2003) Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. Plant Cell 15:1212–1226

FAO and WHO (2009) Benefits and risks of the use of chlorine-containing disinfectants in food production and food processing. Report of a joint FAO/WHO expert meeting, Ann Arbor. http://www.fao.org/docrep/012/i1357e/i1357e.pdf

Friedrich L, Vernooij B, Gaffney T, Morse A, Ryals J (1995) Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. Plant Mol Biol 29:959–968

geNorm (2008) manual. http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf

Gómez-López VM (2012) Selected techniques to decontaminate minimally processed vegetables. In: Bath R, Alias AK, Paliyath G (eds) Progress in food preservation, vol 1. Wiley-Blackwell, London, pp 3–21

Görlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessmann H, Ryals J (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8:629–643

Gozzo F (2003) Systemic acquired resistance in crop protection: from nature to a chemical approach. J Agric Food Chem 51: 4487–4503

Grüner R, Pfitzner UM (1994) The upstream region of the gene for the pathogenesis-related protein 1a from tobacco responds to environmental as well as to developmental signals in transgenic plants. Eur J Biochem 220:247–255

Hammond-Kosack KE, Parker JE (2003) Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. Curr Opin Biotechnol 14:177–193

Heil M, Bostock RM (2002) Induced systemic resistance (ISR) against pathogens in the context of induced plant defenses. Ann Bot 89:503–512

Huang Y-R, Hung Y-C, Hsu S-Y, Huang Y-W, Hwang D-F (2008) Application of electrolyzed water in the food industry. Food Control 19:329–345

ISO 7393-2 (2002) Water quality: determination of free chlorine and total chlorine. Part 2: Colorimetric method using N,N-diethyl-1,4-phenylenediamine, for routine control purposes. International Organization for Standardization (ISO)

Jabs T, Dietrich RA, Dangl JL (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science 27:1853–1856

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Klebanoff SJ (2005) Myeloperoxidase: friend and foe. J Leukoc Biol 77:598–625

Len SV, Hung YC, Erickson M, Kim C (2000) Ultraviolet spectrophotometric characterization and bactericidal properties of electrolyzed oxidizing water as influenced by amperage and pH. J Food Prot 63:1534–1537

Mukhopadhyay S, Ramaswamy R (2012) Application of emerging technologies to control Salmonella in foods: a review. Food Res Int 45:666–677

Niks RE, Marcel TC (2009) Nonhost and basal resistance: how to explain specificity? New Phytol 182:817–828

Ozaki M, Ohshima T, Mukumoto M, Konishi H, Hirashita A, Maeda N, Nakamura Y (2012) A study for biofilm removing and antimicrobial effects by microbubbled tap water and other functional water, electrolyzed hypochlorite water and ozonated water. Dent Mater J 31:662–668

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308–316

Prilutsky VI, Bakhir VM (1997) Electrochemically actuating water: anomalous characteristics, mechanism of biological action. VNIIIMT, Moscow

Sels J, Mathys J, De Coninck BM, Cammue BP, De Bolle MF (2008) Plant pathogenesis-related (PR) proteins: a focus on PR peptides. Plant Physiol Biochem 46:941–950

Shah J (2009) Plants under attack: systemic signals in defence. Curr Opin Plant Biol 12:459–464

Shah J, Chaturvedi R, Chowdhury Z, Venables B, Petros RA (2014) Signaling by small metabolites in systemic acquired resistance. Plant J 79:645–658

Thordal-Christensen H, Zhangt Z, Wei V, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 11:1187–1194

Thorn RMS, Lee SWH, Robinson GM, Greenman J, Reynolds DM (2012) Electrochemically activated solutions: evidence for antimicrobial efficacy and applications in healthcare environments. Eur J Clin Microbiol Infect Dis 31:641–653

Tuzun S, Somanchi A (2006) The possible role of PR proteins in multigenic and induced systemic resistance. In: Tuzun S, Bent E (eds) Multigenic and induced systemic resistance in plants, vol 6. Springer, New York, pp 112–142

Van Loon LC, Rep M, Pieterse CM (2006) Significance of inducible defense-related proteins in infected plants. Ann Rev Phytopathol 44:135–162

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT–PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:research0034.1-research0034.11

Vitha S, Beneš K, Phillips JP, Gartland KMA (1995) Histochemical GUS analysis. In: Gartland KMA, Davey MR (eds) Agrobacterium protocols. Humana Press, Totowa, pp 185–193

Willems E, Leyns L, Vandesompele J (2008) Standardization of real-time PCR gene expression data from independent biological replicates. Anal Biochem 379:127–129

Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochem J 322:681–692

Xiong K, Liu H-J, Liu R, Li L-T (2010) Differences in fungicidal efficiency against *Aspergillus flavus* for neutralized and acidic electrolyzed oxidizing waters. Int J Food Microbiol 137:67–75