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Potato Virus X (PVX) Elimination as Short and Long Term Effects of Hydrogen Peroxide and Salicylic Acid Is differentially Mediated by Oxidative Stress in Synergism with Thermotherapy

Miguel Aguilar-Camacho[1] · Martha E. Mora-Herrera[2] · Humberto A. López-Delgado[1]

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Abstract Short and long term effects of hydrogen peroxide (H_2O_2) and salicylic acid (SA) were examined in: thermotolerance, virus X (PVX)-free microplants obtained by in vitro thermotherapy, catalase activity (CAT) and H_2O_2 concentration. Short term effects (STE) were tested as follows: (a) nodal explants were cultivated 30 d on MS medium containing SA 10^{-5} or 10^{-6} M; and (b) nodal explants were waterlogged for 1 h in 1 or 5 mM H_2O_2 solution and subsequently cultured in MS for 30 d. Long term effects were tested as follows (LTE): the experimental regime was identical to STE but SA and H_2O_2 treated plants were subcultured for an additional 30 d period on MS. All treatments were followed by thermotherapy (32–42 C) for 35 d. Results showed SA and H_2O_2 induced thermotolerance during thermotherapy. The percentage of PVX-free plants obtained in H_2O_2 was significantly higher than in SA in STE and LTE by 3-and 4 fold respectively. CAT activity was differentially mediated by SA and H_2O_2.

Resumen Se estudiaron los efectos del peróxido de hidrógeno (H_2O_2) y ácido salicílico (SA) a corto y largo plazo en: termotolerancia, microplantas libres de virus X (PVX) obtenidas por termoterapia in vitro, actividad catalasa (CAT) y contenido de H_2O_2. Efectos a corto plazo (ECP) se estudiaron como sigue: (a) explantes nodales fueron cultivados 30 d en medio MS con 10^{-5} o 10^{-6} M, y (b) explantes nodales fueron inundados 1 h en soluciones 1 mM o 5 mM de H_2O_2 y después se cultivaron en MS por 30 d. Efectos a largo plazo (ELP) se estudiaron como sigue: el protocolo experimental fue idéntico a ECP pero las plantas tratadas con SA y H_2O_2 fueron subcultivadas por un periodo adicional de 30 d en MS. Todos los tratamientos fueron seguidos por termoterapia (32–42 C) por 35 d. Los resultados mostraron que SA y H_2O_2 indujeron termotolerancia durante termoterapia. El porcentaje de plantas libres de PVX obtenido en H_2O_2 fue significativamente mayor que en SA en ECP y ELP en 3 y 4 veces respectivamente. La actividad CAT fue mediada diferencialmente por SA y H_2O_2.

Keywords Thermotherapy · Reactive oxygen species

Introduction

Potato virus X (PVX) is distributed worldwide in potato growing regions. The viral pathogen is transmitted in several Solanaceae crops by means not involving a vector, i.e., contact of infected with healthy plants (Falcioni et al. 2014). It is well known production of basic and certified potato seed requires virus-free material by in vitro multiplication (Faccioli and Colombarini 1996). Thermotherapy is the most common method for virus eradication, as virus concentration can be reduced when plants are heat-treated. Thermotherapy temperature and time application depend on the virus present and cultivar sensitivity to heat (Rosenberg 2000).

Plants under stressful conditions, such as heat, suffer oxidative stress, which increases the production of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen

✉ Humberto A. López-Delgado
lopez.humberto@inifap.gob.mx

[1] Laboratorio de Fisiología-Biotecnología, Programa Nacional de Papa, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Conjunto SEDAGRO, Metepec, Estado de México C.P. 52140, México

[2] Centro Universitario Tenancingo Universidad Autónoma del Estado de México, Carretera Tenancingo-Villa Guerrero Km 1.5, Estado de México C. P. 52400, México

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peroxide (H_2O_2) (Larkindale and Huang 2004; Shi et al. 2006; Ahmad et al. 2008; Hayat et al. 2010). ROS are toxic at high concentrations, however at moderate levels, ROS can serve as signal molecules, particularly for H_2O_2, which triggers tolerance against various other stress factors (Zhou et al. 2012). H_2O_2 is the most stable ROS, due to its poor reactivity with most organic molecules. It readily diffuses across cell membranes, and reaches distant locations from its primary production sites (Slezak et al. 2007; Kuzniak and Urbanek 2000). Agarwal et al. (2005) reported when H_2O_2 was produced under stress conditions, the molecule acted as an oxidative stress agent and a regulator of various antioxidant enzymes involved in oxidative stress amelioration.

Plants have evolved non-enzymatic and enzymatic antioxidant systems (Larkindale and Huang 2004). Enzymatic systems, such as superoxide dismutase (SOD), peroxidases (POX), and catalases (CAT) protect by directly scavenging ROS radicals, transforming these radicals into less reactive species (Suzuki and Mittler 2006; Mahmdi et al. 2010). However, the reduction of H_2O_2 to H_2O and O_2 can occur without participation of a reducing agent, such as catalase dismutase (Willekens et al. 1995; Mahmdi et al. 2010). Kuzniak and Urbanek (2000) demonstrated SA inhibited this enzyme.

SA belongs to phenolic compounds bearing a hydroxyl group (An and Mou 2011; Vlot et al. 2009). Hayat et al. (2010) reported this molecule was capable of mediating antioxidant system efficiency in plants. External SA application induced protection against several stressors, including long-term drought (Kadioglu et al. 2011), heavy metals (Krantev et al. 2008), and heat (López-Delgado et al. 1998; Dat et al. 1998a, b; He et al. 2005; Shi et al. 2006).

We previously demonstrated SA's capability to induce thermotolerance during thermotherapy and subsequent PVX eradication in infected *S. tuberosum* (potato) microplants (López-Delgado et al. 2004). Induction of heat shock and chilling tolerance by SA and H_2O_2 (López-Delgado and Scott 1997; López-Delgado et al. 1998; Mora-Herrera et al. 2005), as well as microtuberization (López-Delgado et al. 2012) was previously reported.

Because SA and H_2O_2 induced similar plant responses, and SA enhanced thermotolerance and the number of virus-free plants obtained, we hypothesized H_2O_2 could also induce similar thermotolerance and virus cleaning responses throughout thermotherapy. Information regarding the long-term effects of SA and H_2O_2 in thermotolerance and virus cleaning is scarce. Therefore, our objectives were as follows: i) evaluate the short and long term effects of H_2O_2 and SA in thermotolerance induction and virus-free microplants obtained; and ii) analyze the relationship between CAT activity and H_2O_2 content.

Materials and Methods

Plant Material and Culture Conditions

Solanum tuberosum L. microplants clone 040138 only PVX positive were obtained from the National Potato Program of the National Institute for Agriculture and Livestock Research (INIFAP), in Toluca, Mexico and used in all experiments. ELISA confirmed PVX virus presence. Microplants were micropropagated following the methods of Espinoza et al. (1986). Every 30 d, axillary buds were subcultured in jars on MS propagation medium (Murashige and Skoog 1962) at 18 ± 1 C with a 16 h photoperiod at low radiance (fluorescent lights, 35 μmol m^{-2} s^{-1}, 400–700 nm).

Short Term Effects (STE)

SA Treatments Single leafless nodes were cultivated for 30 d on MS propagation medium ± SA (10^{-5}, 10^{-6} M). These concentrations were selected based on previous reports, where SA was effective in enhancing thermotolerance and elimination of potato virus (López-Delgado et al. 2004).

H_2O_2 Treatments Single leafless nodes were placed in petri dishes containing MS propagation medium to avoid dehydration, then saturated for 1 h under sterile conditions in H_2O_2 solution (1.0 and 0.5 mM) or in distilled H_2O (control) at pH 5.6. Explants were subsequently rinsed with sterile distilled H_2O (pH 5.6) and cultured in tubes with MS propagation medium as described for 30 d.

Long-Term Effects (LTE)

The same SA and H_2O_2 concentrations used in STE were applied in LTE experiments.

SA Treatments Same methods as the STE, except microplants were grown an additional 30 d on MS without SA for a total of 30 d on SA followed of 30 d without SA before thermotherapy.

H_2O_2 Treatments Same methods as the STE, except microplants were subcultured an additional 30 d period for a total of 60 d on MS medium before thermotherapy.

Thermotherapy Five days prior to thermotherapy, 30 single nodes per treatment (SA and H_2O_2) were subcultured in tubes with MS propagation medium. Following subculture, thermotherapy was applied in a Biotronette Mark III (Lab Line) environmental chamber under fluorescent lights (35 μmol m^{-2} s^{-1}, 400–700 nm). An alternating temperature regime was performed every day for 35 d. Temperature

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applications alternated between 32 ± 1 C for 23 h and 42 ± 1 C for 1 h.

One day following thermotherapy, survival was evaluated and microplants were subcultured to MS propagation medium. After an additional 30 d, survival assessment and ELISA were conducted to test for PVX.

CAT and H_2O_2 Measurements

Shoot samples for CAT activity or H_2O_2 content were obtained immediately after the short and long term SA and H_2O_2 treatments. For H_2O_2 treatments, samples were collected 5 d following H_2O_2 immersion. All samples were stored in liquid nitrogen until assessed.

Catalase Activity (CAT)

Soluble protein from microplant tissue samples (0.1 g) was extracted by homogenizing the tissue powder in 0.4 mL extraction buffer (50 mM potassium phosphate buffer pH 7.2, 5 mM dithiothreitol (DTT), 1 mM EDTA, and 1 % (*w*/*v*) PVP). CAT activity followed the method of Aebi (1984). The total reaction mixture (3 mL) contained 50 mM potassium, sodium phosphate pH 7.0, and 20 μL of enzyme extract. The addition of 30 mM H_2O_2 initiated the reaction. Decomposition was followed directly by an absorbance decrease at 240 nm every 20 s for 3 min at 26 C. Protein content was determined directly using a spectrophotometer nanodrop 1000 (Thermo Scientific).

H_2O_2 Measurement

Hydrogen peroxide was measured in microplants with roots removed. Tissue samples (~ 0.2 g) were extracted in 1.2 mL ice-cold 5 % (*w*/*v*) trichloroacetic acid (TCA). Following centrifugation (10 min, 10,000 g), 0.5 mL of the supernatant fraction was passed through Dowex-1 resin (0.5 g, Fluka) followed by 3.5 mL 5 % TCA. H_2O_2 was measured in the eluates using luminol-dependent chemiluminescence (Warm and Laties 1982): 0.5 mL of eluate was added to 0.5 mL 0.5 mM luminol (Sigma); this volume was composed of 4.5 mL 0.2 M NH_4OH (pH 9); a 0.452 mL mixture was analyzed in a polystyrene tube (12 × 75 mm, Fisher) using an Optocomp P luminometer (MGM Instruments, USA). Chemiluminescence was initiated by injecting 50 μL of 0.5 mM potassium ferricyanide in 0.2 M NH_4OH and emitted photons were counted over 5 s. A parallel sample of each initial extract was processed after addition of a known H_2O_2 concentration to provide a recovery correlation factor.

Elisa

PVX presence was confirmed by the Enzyme-Linked Immunosorbent Assay (ELISA) method (Clark and Adams 1977). For more accurate detection, ELISA was performed sampling meristem, stem and leaf tissues included (~ 0.3 g), before SA and H_2O_2 treatments and 30 d after thermotherapy. PVX virus presence was recorded using a Multiskcan FC spectrophotometer (Thermo Scientific).

Statistical Analysis

Significant results were tested using Analysis of Variance (ANOVA) and *post hoc* Duncan's Multiple Range Test (Duncan 1955). The a priori significance level was established at $P < 0.05$. All experiments were performed with three replicates.

Results

Short-Term Effects (STE)

Both SA concentrations (10^{-5}, 10^{-6} M) showed significantly increased PVX-infected microplant survival to thermotherapy (Fig. 1a). Similarly, SA treatment explant H_2O_2 saturation induced a significantly longer survival increment following thermotherapy compared with the control (Fig. 1b). However, neither SA nor H_2O_2 induced significant differences in recovery survival after thermotherapy (data not shown). SA and H_2O_2 strongly induced thermotolerance during thermotherapy.

Treated microplant CAT activity and H_2O_2 content were assessed prior to thermotherapy. SA 10^{-6} M significantly increased CAT activity compared with the control (Fig. 2a); whereas, results did not detect significant differences in H_2O_2 (1.0 and 0.5 mM) treatment on CAT activity induction (data not shown). However, SA (10^{-6} M) resulted in a significant reduction in H_2O_2 concentration (Fig. 2b).

Percent PVX-free microplants were determined. Treatments of both molecules significantly improved the percentage of PVX-free microplants compared with the control, however H_2O_2 induced a higher virus-free plant percentage than SA at the tested concentrations. SA treatments significantly increased PVX-free microplants by 1.9 and 1.5 fold for 10^{-6} and 10^{-5} M, respectively relative to the control (Fig. 3a), whereas H_2O_2 treatments enhanced PVX-free microplants by 1.5 and 3-fold for 0.5 and 1.0 mM, respectively relative to the control (Fig. 3b). These results supported our observations that SA or H_2O_2 induced thermotolerance and improved PVX-free microplants during thermotherapy.

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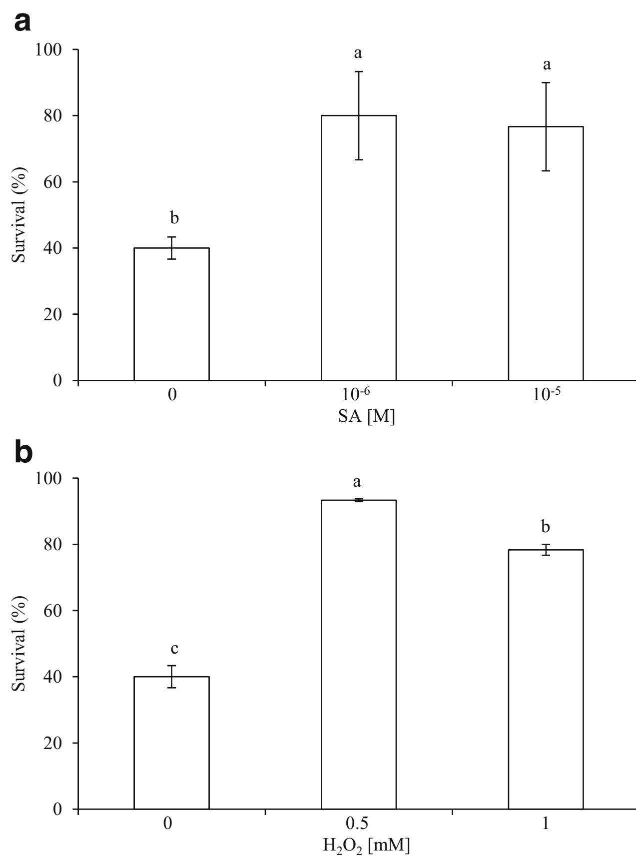

Fig. 1 Short term effect of SA and H_2O_2 on tolerance to thermotherapy (32–42 C) of potato microplants clone 040138. **a** Microplants from SA treatment. **b** Microplants from H_2O_2 treatment. Survival of microplants was assessed one day after thermotherapy (n = 30). Data are means of 3 experiments ± S.E. Bars labeled with same letter were not significantly different by ANOVA and Duncan test (α = 0.05)

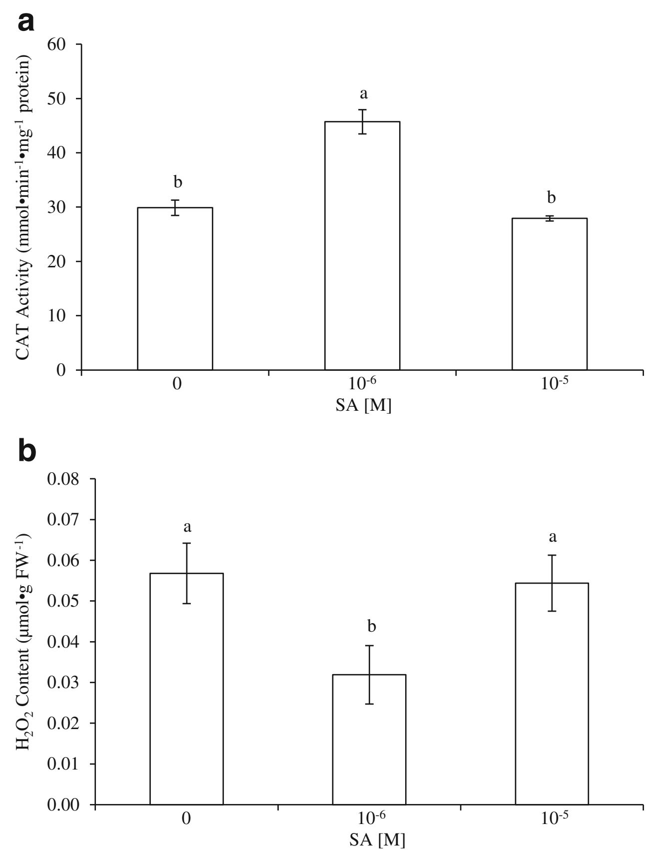

Fig. 2 Short term effect of SA and H_2O_2 on catalase activity (**a**) and hydrogen peroxide content (**b**) of potato microplants clone 040138. Data are means ± S.E. bars of three experiments (n = 3), each one with three samples, measured in triplicate. Bars labeled with same letter were not significantly different by ANOVA and Duncan test (α = 0.05)

Long-Term Effects (LTE)

Long term SA and H_2O_2 effects were determined. Similar to STE, SA and H_2O_2 LTE significantly increased survival length post thermotherapy in PVX-infected microplants (Fig. 4a, b). These results supported our original hypothesis that SA and H_2O_2 effected thermotolerance, even though application had ceased. However, neither SA nor H_2O_2 induced significant differences in recovery survival following thermotherapy (data not shown). Both SA concentrations (10^{-5}, 10^{-6} M) enhanced survival up to 1.5 fold, while H_2O_2 increased survival in 1.4 and 1.5 fold compared to the control in 0.5 and 1 mM, respectively.

CAT activity and H_2O_2 content in treated microplants were assessed prior to thermotherapy. H_2O_2 long-term effects generated H_2O_2 internal content changes in microplants. CAT enzymatic activity was significantly enhanced under H_2O_2 treatment compared with the control (Fig. 5a) in 1.2 and 1.3 fold for 0.5 and 1 mM H_2O_2, respectively. SA did not induce significant effects on CAT activity (data not shown).

Interestingly, SA significantly reduced H_2O_2 concentration relative to the control (Fig. 5b); 10^{-6} M reduced H_2O_2 concentration in 1.3 fold and 10^{-5} M in 1.4 fold.

An increased microplant H_2O_2 internal concentration was induced by H_2O_2 via a dose-response (Fig 5c); results showed 0.5 mM enhanced H_2O_2 concentration in 1. 7 fold and 1.0 mM by 2.2-fold.

Similar to STE, SA and H_2O_2 significantly increased the percentage of virus-free microplants during LTE. PVX-free microplants increased by 2 fold under SA 10^{-6} M treatment compared with the control, whereas under SA 10^{-5} M, PVX-free microplants increased by 1.7 fold (Fig 6a). In addition, an increased number of PVX-free microplants was observed following H_2O_2 treatment. Results showed 4- and 3.3-fold were respectively observed in 0.5 and 1.0 mM H_2O_2 relative to the control (Fig. 6b).

Discussion

The results of this study demonstrated SA and H_2O_2 induced similar effects on thermotolerance during thermotherapy and

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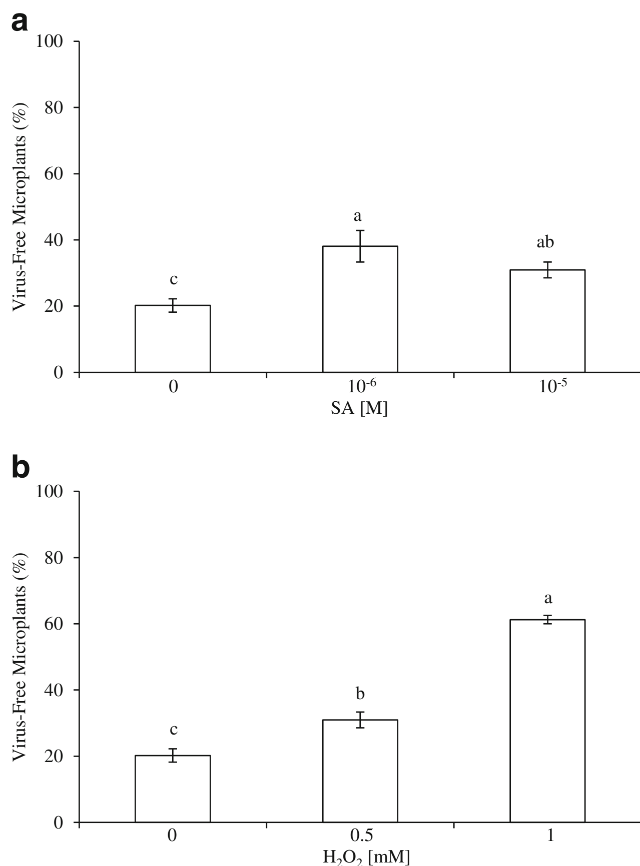

Fig. 3 Short term effect of SA and on the percentage of PVX-free microplants of potato clone 040138 subcultured to MS medium from in vitro thermotherapy- treated plants. **a** Microplants from SA treatment. **b** Microplants from H_2O_2 treatment. Virus presence was determined by ELISA test. Data are means of 3 experiments (*n* = 8–27). Bars labeled with same letter were not significantly different by ANOVA and Duncan test (α = 0.05)

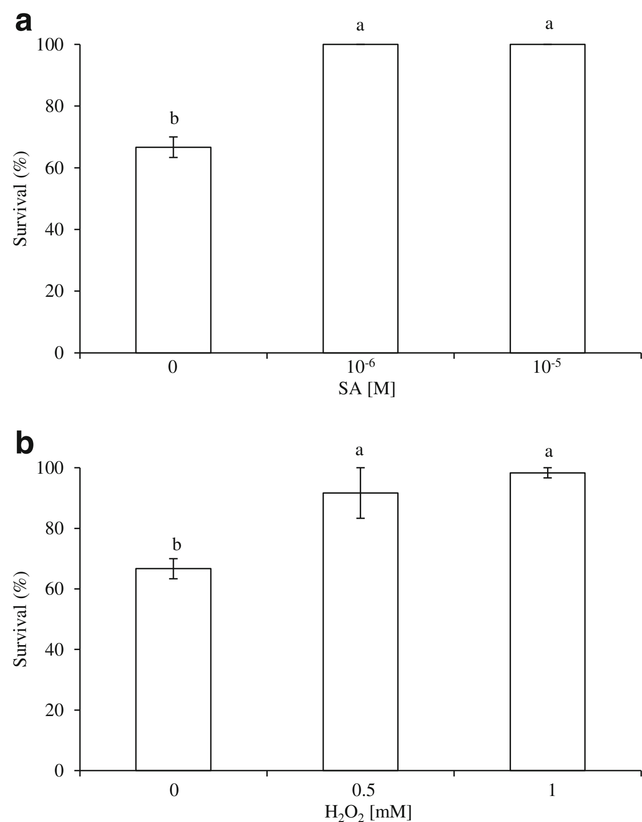

Fig. 4 Long term effect of SA and H_2O_2 on resistance to thermotherapy (32–42 C) of potato microplants clone 040138. **a** Microplants from SA treatment. **b** Microplants from H_2O_2 treatment. Survival of microplants was assessed one day after thermotherapy (*n* = 30). Data are means of 3 experiments ± S.E. Bars labeled with same letter were not significantly different by ANOVA and Duncan test (α = 0.05)

the generation of PVX-free plants in the short and long terms. The physiological importance of interactions between these two signaling molecules remains poorly characterized and elucidated. Because former studies reported SA and H_2O_2 induced heat tolerance in potato, the present experiments investigated the short and long term effects of SA and H_2O_2 signaling in potato during thermotherapy. We previously demonstrated short term SA treatment induced thermotolerance during thermotherapy and enhanced the percentage of PVX-free microplants (López-Delgado et al. 2004). In addition, we demonstrated microplant tolerance to heat shock was induced following soaking in H_2O_2 (López-Delgado et al., 1998). Reports of H_2O_2 treatment effects on virus-free plant generation are not available and similarly long-term effects of SA and H_2O_2 on thermotolerance and virus cleaning by thermotherapy have not been reported. However, heat tolerance by SA treatment was exhibited in many species, (Dat et al. 1998a, b; Senaratna et al. 2000; Larkindale and Knight 2002; Larkindale and Huang 2004; Chakraborty and Tongden 2005; Wang and Li 2006; Pan et al. 2006; He et al. 2005; Shi et al. 2006).

H_2O_2 and SA induced thermotolerance in short and long terms, which increased survival percentages after thermotherapy. Comparable heat tolerance induction by H_2O_2 was reported (Uchida et al. 2002; Larkindale and Huang 2004; López-Delgado et al. 1998; Wang et al. 2014). Bearing in mind H_2O_2's function as a signaling molecule, we investigated the potential role of H_2O_2 in plant thermotherapy, and considered our primary goals: heat tolerance and virus eradication. We recognized achieving our objectives would impose stressful conditions on plants. The potential application of SA and H_2O_2 in thermotherapy was demonstrated, since potato genotypes do not often survive long periods of heat during thermotherapy and heat tolerance is not automatically associated with virus eradication.

Interestingly, 1.0 mM H_2O_2 increased PVX-free plants and showed similar short (3-fold higher, Fig. 3b) and long-term (3.3-fold higher, Fig. 6b) results compared with the control. However, a lower H_2O_2 concentration (0.5 mM) in the long term induced higher values of PVX-free microplants (4-fold, Fig. 6b) relative to the control.

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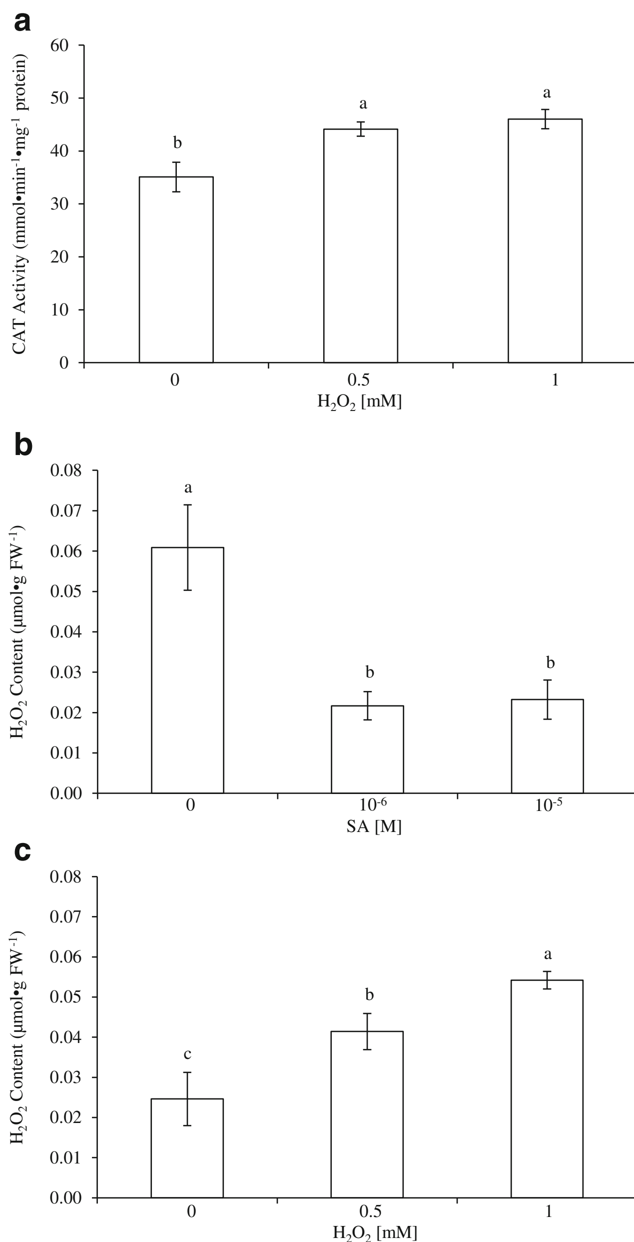

Fig. 5 Long term effect of SA and H_2O_2 on CAT activity and H_2O_2 content of potato microplants clone 040138. **a, c** Microplants from H_2O_2 treatment. **b** Microplants from SA treatment. Data are means ± S.E. bars of three experiments (*n* = 3), each one with three samples measured in triplicate. Bars labeled with same letter were not significantly different by ANOVA and Duncan test (α = 0.05)

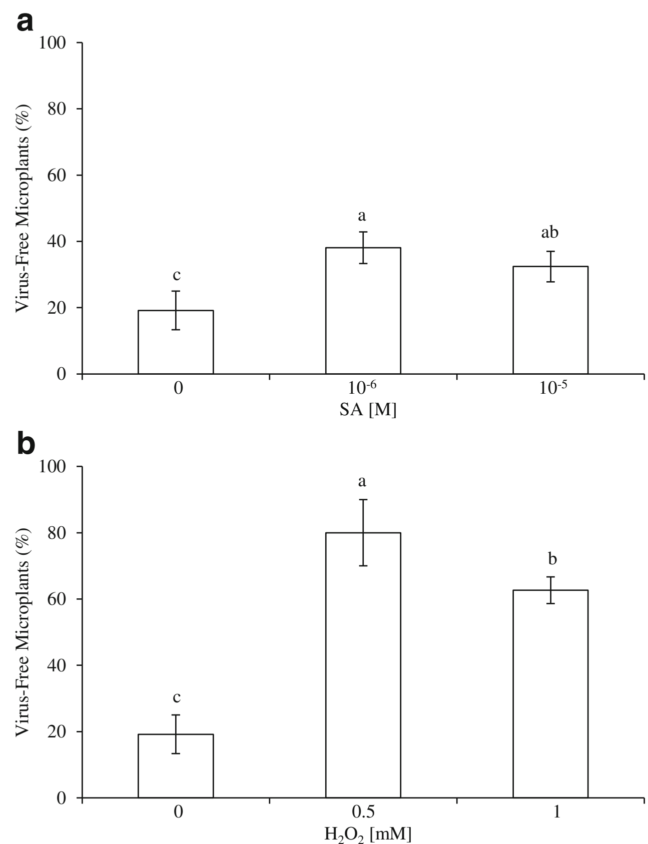

Fig. 6 Long term effect of SA and H_2O_2 on the percentage of PVX-free microplants of potato clone 040,138 subcultured to MS medium from in vitro thermotherapy- treated plants. **a** Microplants from SA treatment. **b** Microplants from H_2O_2 treatment. Virus presence was determined by ELISA test. Data are means of 3 experiments (*n* = 8–24). Bars labeled with same letter were not significantly different by ANOVA and Duncan test (α = 0.05)

We formerly found increased internal H_2O_2 concentrations before heat shock (López-Delgado et al. 1998) or thermotherapy in potato (López-Delgado et al. 2004) was associated with survival augmentation and this increased internal H_2O_2 concentration was associated with reduced CAT activity mediated by SA. The results of our current study were consistent with these reports; significant internal H_2O_2 increases were observed under H_2O_2 LTE (Fig. 5b). A rise in microplant internal H_2O_2 concentration can be associated with significantly higher survival and more PVX-free plants, compared with the control. However, the effects of SA on H_2O_2 accumulation differ depending on potato cultivar (López-Delgado et al. 2004).

SA and H_2O_2 under short and long term treatments significantly increased the percentage of PVX-free plants, however, it is notable the percentage of virus-free plants was greater under H_2O_2 (Figs 3b, 6b) relative to SA (Figs 3a, 6a) treatments, particularly for LTE.

Furthermore, enhanced CAT activity under SA 10^{-6} M treatment during STE (Fig. 2a) was observed, which was associated with a higher percentage of PVX-free plants (Fig. 3a). Clarke et al. (2002) reported SA treatments in bean plants showed elevated CAT activity, which inhibited virus replication, congruent with our results. In addition, treatments increasing the capacity of plants to scavenge ROS might hinder virus replication (Clarke et al. 2002).

Interestingly, SA 10^{-6} M treatment increased CAT activity during STE (Fig. 2a) and reduced microplant H_2O_2 internal concentration (Fig. 2b). These results exhibited a lack of

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congruency with former reports in potato, where SA reduced CAT activity leading to H_2O_2 internal accumulation during heat shock (López-Delgado et al. 1998), thermotherapy (López-Delgado et al. 2004), freezing tolerance induction (Mora-Herrera et al. 2005), and phytoplasma-associated stress protection (Sanchez-Rojo et al. 2011). This observation was not necessarily in conflict, if we assume the following: SA induction by H_2O_2 and catalase inactivation by SA function cooperatively in a cyclic mechanism to amplify the SA/H_2O_2 signal (Chamnongpol et al. 1996; Leon et al. 1995). In this work, microplant increased thermotherapy survival and increased percentage of PVX-free microplants compared with control microplants, particularly at SA 10^{-6} M (Fig. 3a).

The literature documents a complex relationship between SA and H_2O_2 signaling in plants; Dat et al. (2000) showed SA increased H_2O_2 and Chamnongpol et al. (1996) reported SA was induced by H_2O_2. Oxidative stress physiology during thermotherapy is a specific research area, and an even more specialized topic is examined in the relationship between SA and H_2O_2 signaling responses mediated by two combined stress factors, high temperature and viral infection.

Conclusions

The percentage of PVX-free plants obtained under H_2O_2 was significantly higher than under SA conditions in short and long term treatment effects. Although SA and H_2O_2 induced similar thermotolerance responses and generated a higher percentage of PVX-free microplants, SA and H_2O_2 act via different signaling pathways, as suggested for induction of potato freezing tolerance (Mora-Herrera et al. 2005). The accurate measurement of ROS changes in response to thermotherapy-virus infection-SA/H_2O_2 treatment warrants further investigation to better understand the oxidative stress physiology involved in thermotherapy and eventually gain more efficient virus eradication protocols.

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