

*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 Thermootherapy*

**Miguel Aguilar-Camacho, Martha E. Mora-Herrera & Humberto A. López-Delgado**

**American Journal of Potato Research**  
The Official Journal of the Potato Association of America

ISSN 1099-209X

Am. J. Potato Res.  
DOI 10.1007/s12230-016-9509-5



**Your article is protected by copyright and all rights are held exclusively by The Potato Association of America. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at [link.springer.com](http://link.springer.com)".**

# 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<sup>1</sup> · Martha E. Mora-Herrera<sup>2</sup> · Humberto A. López-Delgado<sup>1</sup>

© The Potato Association of America 2016

**Abstract** Short and long term effects of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and salicylic acid (SA) were examined in: thermotolerance, virus X (PVX)-free microplants obtained by in vitro thermotherapy, catalase activity (CAT) and H<sub>2</sub>O<sub>2</sub> concentration. Short term effects (STE) were tested as follows: (a) nodal explants were cultivated 30 d on MS medium containing SA 10<sup>-5</sup> or 10<sup>-6</sup> M; and (b) nodal explants were waterlogged for 1 h in 1 or 5 mM H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> induced thermotolerance during thermotherapy. The percentage of PVX-free plants obtained in H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>.

**Resumen** Se estudiaron los efectos del peróxido de hidrógeno (H<sub>2</sub>O<sub>2</sub>) 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<sub>2</sub>O<sub>2</sub>. Efectos a corto plazo (ECP) se

estudiaron como sigue: (a) explantes nodales fueron cultivados 30 d en medio MS con 10<sup>-5</sup> o 10<sup>-6</sup> M, y (b) explantes nodales fueron inundados 1 h en soluciones 1 mM o 5 mM de H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> indujeron termotolerancia durante termoterapia. El porcentaje de plantas libres de PVX obtenido en H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>.

**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 (<sup>1</sup>O<sub>2</sub>), superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl radical (OH<sup>-</sup>), and hydrogen

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

<sup>1</sup> 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

<sup>2</sup> Centro Universitario Tenancingo Universidad Autónoma del Estado de México, Carretera Tenancingo-Villa Guerrero Km 1.5, Estado de México C. P. 52400, México

peroxide ( $\text{H}_2\text{O}_2$ ) (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  $\text{H}_2\text{O}_2$ , which triggers tolerance against various other stress factors (Zhou et al. 2012).  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{O}_2$  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  $\text{H}_2\text{O}_2$  (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  $\text{H}_2\text{O}_2$  induced similar plant responses, and SA enhanced thermotolerance and the number of virus-free plants obtained, we hypothesized  $\text{H}_2\text{O}_2$  could also induce similar thermotolerance and virus cleaning responses throughout thermotherapy. Information regarding the long-term effects of SA and  $\text{H}_2\text{O}_2$  in thermotolerance and virus cleaning is scarce. Therefore, our objectives were as follows: i) evaluate the short and long term effects of  $\text{H}_2\text{O}_2$  and SA in thermotolerance induction and virus-free microplants obtained; and ii) analyze the relationship between CAT activity and  $\text{H}_2\text{O}_2$  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  $18 \pm 1$  C with a 16 h photoperiod at low radiance (fluorescent lights,  $35 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 400–700 nm).

### Short Term Effects (STE)

**SA Treatments** Single leafless nodes were cultivated for 30 d on MS propagation medium  $\pm$  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).

**$\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  solution (1.0 and 0.5 mM) or in distilled  $\text{H}_2\text{O}$  (control) at pH 5.6. Explants were subsequently rinsed with sterile distilled  $\text{H}_2\text{O}$  (pH 5.6) and cultured in tubes with MS propagation medium as described for 30 d.

### Long-Term Effects (LTE)

The same SA and  $\text{H}_2\text{O}_2$  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.

**$\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$ ) 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 ( $35 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 400–700 nm). An alternating temperature regime was performed every day for 35 d. Temperature



applications alternated between  $32 \pm 1$  C for 23 h and  $42 \pm 1$  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<sub>2</sub>O<sub>2</sub> Measurements

Shoot samples for CAT activity or H<sub>2</sub>O<sub>2</sub> content were obtained immediately after the short and long term SA and H<sub>2</sub>O<sub>2</sub> treatments. For H<sub>2</sub>O<sub>2</sub> treatments, samples were collected 5 d following H<sub>2</sub>O<sub>2</sub> 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  $\mu$ L of enzyme extract. The addition of 30 mM H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>4</sub>OH (pH 9); a 0.452 mL mixture was analyzed in a polystyrene tube (12  $\times$  75 mm, Fisher) using an Optocomp P luminometer (MGM Instruments, USA). Chemiluminescence was initiated by injecting 50  $\mu$ L of 0.5 mM potassium ferricyanide in 0.2 M NH<sub>4</sub>OH and emitted photons were counted over 5 s. A parallel sample of each initial extract was processed after addition of a known H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> treatments and 30 d after thermotherapy. PVX virus presence was recorded using a Multiskan 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.

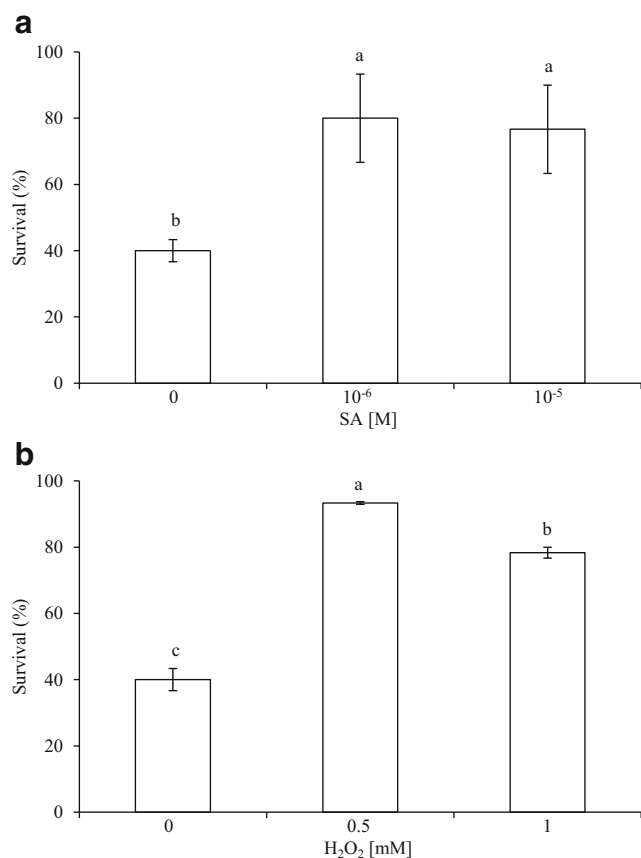
## Results

### 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<sub>2</sub>O<sub>2</sub> saturation induced a significantly longer survival increment following thermotherapy compared with the control (Fig. 1b). However, neither SA nor H<sub>2</sub>O<sub>2</sub> induced significant differences in recovery survival after thermotherapy (data not shown). SA and H<sub>2</sub>O<sub>2</sub> strongly induced thermotolerance during thermotherapy.

Treated microplant CAT activity and H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> (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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> induced thermotolerance and improved PVX-free microplants during thermotherapy.

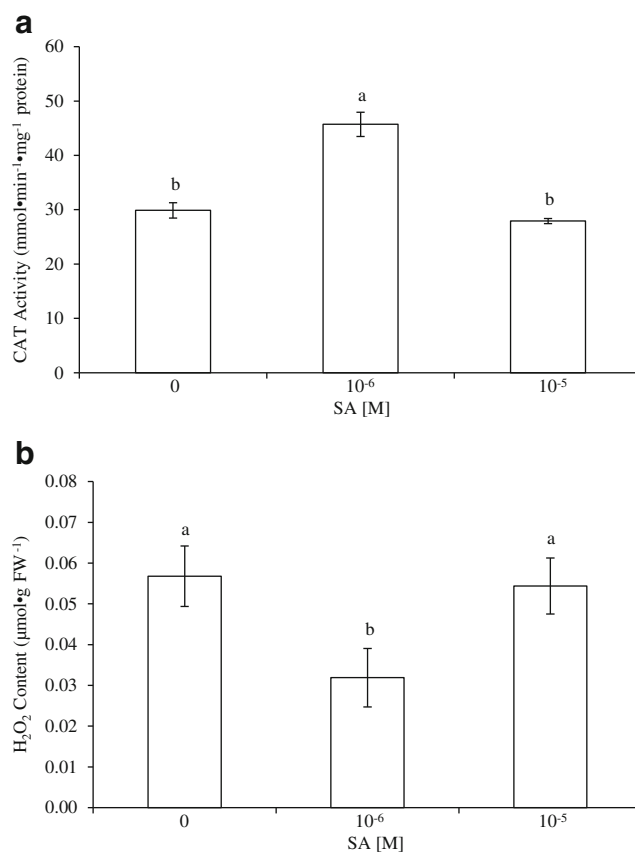


**Fig. 1** Short term effect of SA and H<sub>2</sub>O<sub>2</sub> on tolerance to thermotherapy (32–42 °C) of potato microplants clone 040138. **a** Microplants from SA treatment. **b** Microplants from H<sub>2</sub>O<sub>2</sub> treatment. Survival of microplants was assessed one day after thermotherapy ( $n = 30$ ). Data are means of 3 experiments  $\pm$  S.E. Bars labeled with same letter were not significantly different by ANOVA and Duncan test ( $\alpha = 0.05$ )

### Long-Term Effects (LTE)

Long term SA and H<sub>2</sub>O<sub>2</sub> effects were determined. Similar to STE, SA and H<sub>2</sub>O<sub>2</sub> LTE significantly increased survival length post thermotherapy in PVX-infected microplants (Fig. 4a, b). These results supported our original hypothesis that SA and H<sub>2</sub>O<sub>2</sub> effected thermotolerance, even though application had ceased. However, neither SA nor H<sub>2</sub>O<sub>2</sub> induced significant differences in recovery survival following thermotherapy (data not shown). Both SA concentrations (10<sup>-5</sup>, 10<sup>-6</sup> M) enhanced survival up to 1.5 fold, while H<sub>2</sub>O<sub>2</sub> increased survival in 1.4 and 1.5 fold compared to the control in 0.5 and 1 mM, respectively.

CAT activity and H<sub>2</sub>O<sub>2</sub> content in treated microplants were assessed prior to thermotherapy. H<sub>2</sub>O<sub>2</sub> long-term effects generated H<sub>2</sub>O<sub>2</sub> internal content changes in microplants. CAT enzymatic activity was significantly enhanced under H<sub>2</sub>O<sub>2</sub> treatment compared with the control (Fig. 5a) in 1.2 and 1.3 fold for 0.5 and 1 mM H<sub>2</sub>O<sub>2</sub>, respectively. SA did not induce significant effects on CAT activity (data not shown).



**Fig. 2** Short term effect of SA and H<sub>2</sub>O<sub>2</sub> on catalase activity (**a**) and hydrogen peroxide content (**b**) of potato microplants clone 040138. Data are means  $\pm$  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 ( $\alpha = 0.05$ )

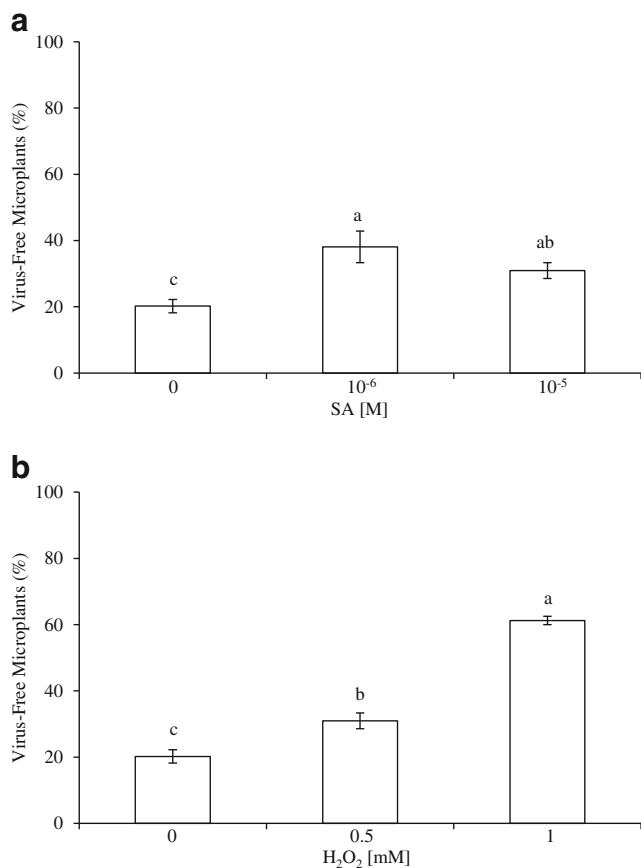
Interestingly, SA significantly reduced H<sub>2</sub>O<sub>2</sub> concentration relative to the control (Fig. 5b); 10<sup>-6</sup> M reduced H<sub>2</sub>O<sub>2</sub> concentration in 1.3 fold and 10<sup>-5</sup> M in 1.4 fold.

An increased microplant H<sub>2</sub>O<sub>2</sub> internal concentration was induced by H<sub>2</sub>O<sub>2</sub> via a dose-response (Fig 5c); results showed 0.5 mM enhanced H<sub>2</sub>O<sub>2</sub> concentration in 1.7 fold and 1.0 mM by 2.2-fold.

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

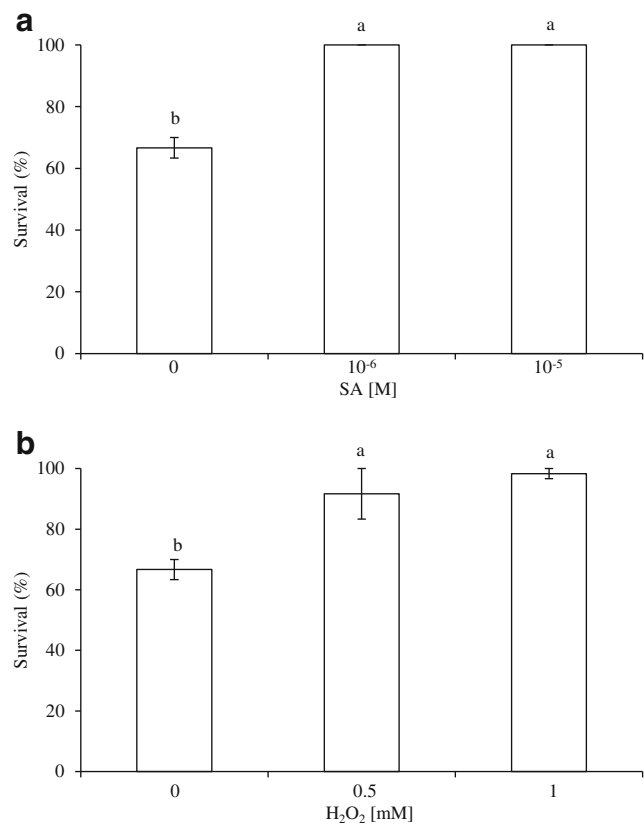
### Discussion

The results of this study demonstrated SA and H<sub>2</sub>O<sub>2</sub> induced similar effects on thermotolerance during thermotherapy and



**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<sub>2</sub>O<sub>2</sub> 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 ( $\alpha = 0.05$ )

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<sub>2</sub>O<sub>2</sub> induced heat tolerance in potato, the present experiments investigated the short and long term effects of SA and H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> (López-Delgado et al., 1998). Reports of H<sub>2</sub>O<sub>2</sub> treatment effects on virus-free plant generation are not available and similarly long-term effects of SA and H<sub>2</sub>O<sub>2</sub> 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

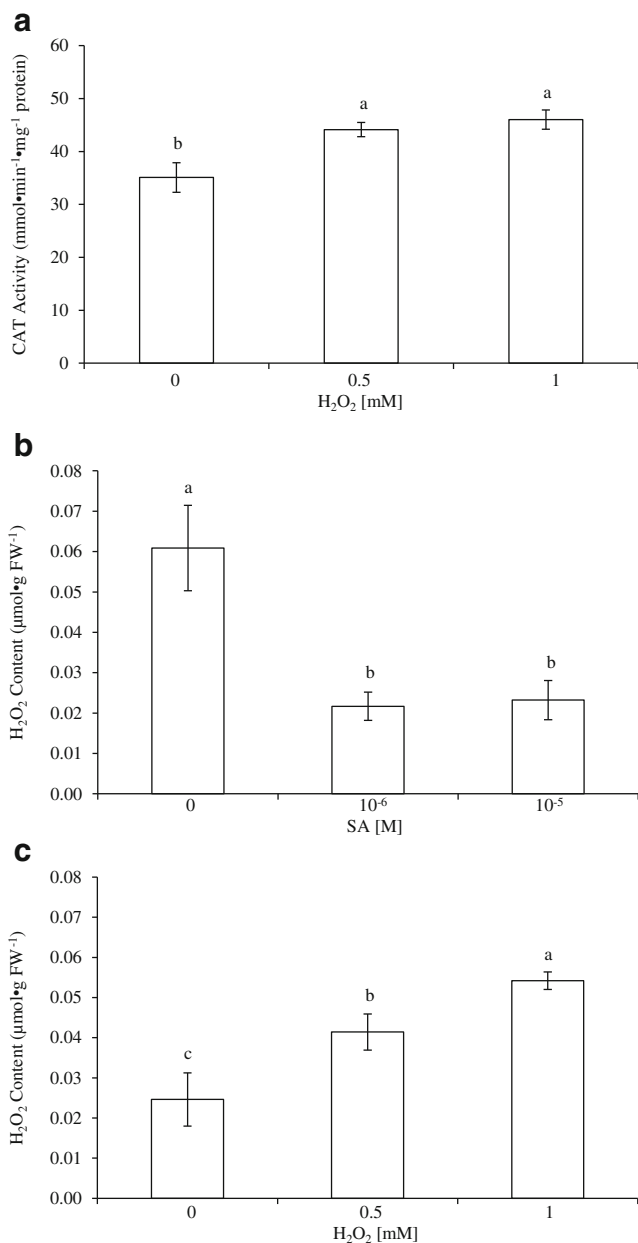


**Fig. 4** Long term effect of SA and H<sub>2</sub>O<sub>2</sub> on resistance to thermotherapy (32–42 C) of potato microplants clone 040138. **a** Microplants from SA treatment. **b** Microplants from H<sub>2</sub>O<sub>2</sub> treatment. Survival of microplants was assessed one day after thermotherapy ( $n = 30$ ). Data are means of 3 experiments  $\pm$  S.E. Bars labeled with same letter were not significantly different by ANOVA and Duncan test ( $\alpha = 0.05$ )

2005; Wang and Li 2006; Pan et al. 2006; He et al. 2005; Shi et al. 2006).

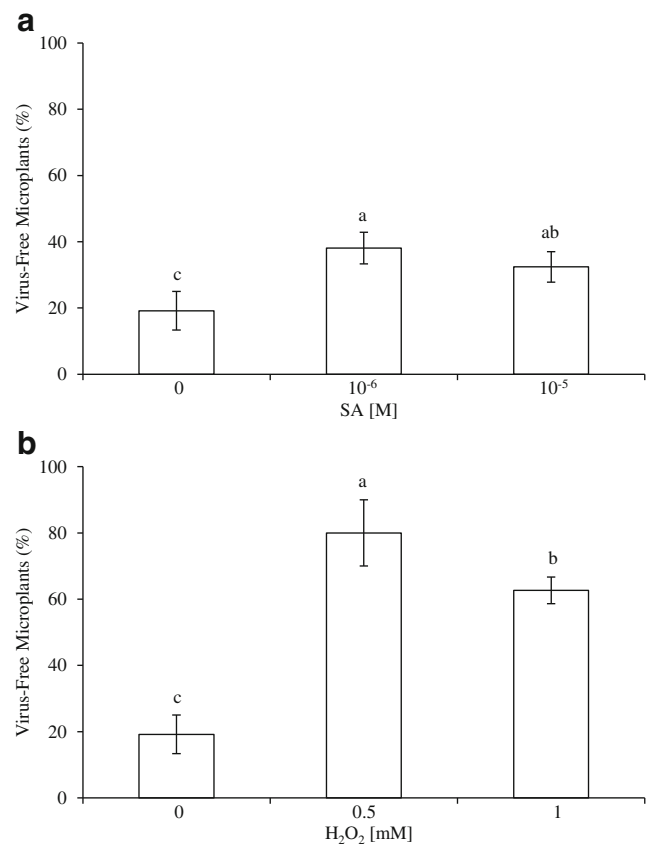
H<sub>2</sub>O<sub>2</sub> and SA induced thermotolerance in short and long terms, which increased survival percentages after thermotherapy. Comparable heat tolerance induction by H<sub>2</sub>O<sub>2</sub> was reported (Uchida et al. 2002; Larkindale and Huang 2004; López-Delgado et al. 1998; Wang et al. 2014). Bearing in mind H<sub>2</sub>O<sub>2</sub>'s function as a signaling molecule, we investigated the potential role of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> concentration (0.5 mM) in the long term induced higher values of PVX-free microplants (4-fold, Fig. 6b) relative to the control.



**Fig. 5** Long term effect of SA and H<sub>2</sub>O<sub>2</sub> on CAT activity and H<sub>2</sub>O<sub>2</sub> content of potato microplants clone 040138. **a, c** Microplants from H<sub>2</sub>O<sub>2</sub> treatment. **b** Microplants from SA treatment. Data are means  $\pm$  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 ( $\alpha = 0.05$ )

We formerly found increased internal H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> concentration was associated with reduced CAT activity mediated by SA. The results of our current study were consistent with these reports; significant internal H<sub>2</sub>O<sub>2</sub> increases were observed under H<sub>2</sub>O<sub>2</sub> LTE (Fig. 5b). A rise in microplant internal H<sub>2</sub>O<sub>2</sub> concentration can be associated with



**Fig. 6** Long term effect of SA and H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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 ( $\alpha = 0.05$ )

significantly higher survival and more PVX-free plants, compared with the control. However, the effects of SA on H<sub>2</sub>O<sub>2</sub> accumulation differ depending on potato cultivar (López-Delgado et al. 2004).

SA and H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> (Figs 3b, 6b) relative to SA (Figs 3a, 6a) treatments, particularly for LTE.

Furthermore, enhanced CAT activity under SA 10<sup>-6</sup> 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<sup>-6</sup> M treatment increased CAT activity during STE (Fig. 2a) and reduced microplant H<sub>2</sub>O<sub>2</sub> internal concentration (Fig. 2b). These results exhibited a lack of



congruency with former reports in potato, where SA reduced CAT activity leading to H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> and catalase inactivation by SA function cooperatively in a cyclic mechanism to amplify the SA/H<sub>2</sub>O<sub>2</sub> signal (Chamngongpol 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<sup>-6</sup> M (Fig. 3a).

The literature documents a complex relationship between SA and H<sub>2</sub>O<sub>2</sub> signaling in plants; Dat et al. (2000) showed SA increased H<sub>2</sub>O<sub>2</sub> and Chamngongpol et al. (1996) reported SA was induced by H<sub>2</sub>O<sub>2</sub>. 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<sub>2</sub>O<sub>2</sub> signaling responses mediated by two combined stress factors, high temperature and viral infection.

## Conclusions

The percentage of PVX-free plants obtained under H<sub>2</sub>O<sub>2</sub> was significantly higher than under SA conditions in short and long term treatment effects. Although SA and H<sub>2</sub>O<sub>2</sub> induced similar thermotolerance responses and generated a higher percentage of PVX-free microplants, SA and H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> treatment warrants further investigation to better understand the oxidative stress physiology involved in thermotherapy and eventually gain more efficient virus eradication protocols.

**Acknowledgments** This research was supported by a grant from Recursos Fiscales, INIFAP.

## References

- Aebi, H. 1984. Catalase in vitro. *Methods in Enzymology* 105: 121–126.
- Agarwal, S., R.K. Sairam, G.C. Srivastava, A. Tyagi, and R.C. Meena. 2005. Role of ABA, salicylic acid, calcium and hydrogen peroxide on antioxidant enzymes induction in wheat seedlings. *Plant Science* 169: 559–570.
- Ahmad, P., M. Sarwat, and S. Sharma. 2008. Reactive oxygen species, antioxidants and signaling in plants. *Journal of Plant Biology* 51: 167–173.
- An, C., and Z. Mou. 2011. Salicylic acid and its function in plant immunity. *Journal of Integrative Plant Biology* 53: 412–428.
- Chakraborty, U., and C. Tongden. 2005. Evaluation of heat acclimation and salicylic acid treatments as patent inducers of thermotolerance in *Cicer arietinum* L. *Current Science* 89: 384–389.
- Chamngongpol, S., H. Willekens, C. Langebartels, M. Van Montagu, D. Inzé, and W.V. Camp. 1996. Transgenic tobacco with a reduced catalase activity develops necrotic lesions and induces pathogenesis-related expression under high light. *The Plant Journal* 10: 491–503.
- Clark, M.F., and A.N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbant assay for the detection of plant viruses. *Journal of General Virology* 34: 475–483.
- Clarke, S.F., P.I. Guy, D.J. Burrit, and P.E. Jameson. 2002. Changes in the activities of antioxidant enzymes in response to virus infection and hormone treatment. *Physiologia Plantarum* 114: 157–164.
- Dat, J.F., C.H. Foyer, and I.M. Scott. 1998a. Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. *Plant Physiology* 118: 1455–1461.
- Dat, J.F., H. López-Delgado, C.H. Foyer, and I.M. Scott. 1998b. Parallel changes in H<sub>2</sub>O<sub>2</sub> and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. *Plant Physiology* 116: 1351–1357.
- Dat, J.F., H. López-Delgado, C.H. Foyer, and I.M. Scott. 2000. Effects of salicylic acid on oxidative stress and thermotolerance in tobacco. *Journal of Plant Physiology* 156: 659–665.
- Duncan, D.B. 1955. Multiple range and multiple F tests. *Biometrics* 11: 1–42.
- Espinoza, N.O., R. Estrada, D. Silva-Rodriguez, P. Tovar, R. Lizarraga, and J.H. Dodds. 1986. The potato: a model crop plant for tissue culture. *Outlook on Agriculture* 15: 21–26.
- Faccioli, G., and A. Colombarini. 1996. Correlation of potato virus S and virus M contents of potato meristem tips with the percentage of virus-free plantlets produced *in vitro*. *Potato Research* 39: 129–140.
- Falconi, T., J.P. Ferrio, A.I. Cueto, J. Giné, M.A. Achón, and V. Medina. 2014. Effect of salicylic acid treatment on tomato plant physiology and tolerance to potato virus X infection. *European Journal of Plant Pathology* 138: 331–345.
- Hayat, Q., S. Hayat, M. Irfan, and A. Ahmad. 2010. Effect of exogenous salicylic acid under changing environment. *Environmental and Experimental Botany* 68: 14–25.
- He, Y., Y. Liu, W. Cao, M. Huai, B. Xu, and B. Huang. 2005. Effects of salicylic acid on heat tolerance associated with antioxidant metabolism in Kentucky bluegrass. *Crop Science* 45: 988–995.
- Kadioglu, A., N. Saruhan, A. Saglam, R. Terzi, and T. Acet. 2011. Exogenous salicylic acid alleviates effects of long term drought stress delays leaf rolling by inducing antioxidant system. *Plant Growth Regulation* 64: 27–37.
- Krantev, A., R. Yordanova, T. Janda, G. Szali, and L. Popova. 2008. Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants. *Journal of Plant Physiology* 165: 920–931.
- Kuzniak, E., and H. Urbanek. 2000. The involvement of hydrogen peroxide in plant responses to stress. *Acta Physiologiae Plantarum* 22: 195–203.
- Larkindale, J., and B. Huang. 2004. Thermotolerance and antioxidant systems in *Agrostis stolonifera*: involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene. *Journal of Plant Physiology* 164: 405–413.
- Larkindale, J., and M.R. Knight. 2002. Protection against heat stress-induced oxidative damage in arabidopsis involves calcium, abscisic acid, ethylene and salicylic acid. *Plant Physiology* 128: 682–695.
- León, J., M.A. Lawton, and I. Raskin. 1995. Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. *Plant Physiology* 108: 1673–1678.
- López-Delgado, H., M.E. Mora-Herrera, H.A. Zavaleta-Mancera, M. Cadena-Hinojosa, and I.M. Scott. 2004. Salicylic acid enhances heat tolerance and potato virus X (PVX) elimination during

- thermotherapy of potato microplants. *American Journal of Potato Research* 81: 171–176.
- López-Delgado, H.A., S. Sánchez-Rojo, M.E. Mora-Herrera, and R. Martínez-Gutiérrez. 2012. Micro-tuberization as a Long Term Effect of Hydrogen Peroxide on Potato Plants. *American Journal of Potato Research* 89: 240–244.
- López-Delgado, H., and I.M. Scott. 1997. Induction of *in vitro* tuberization of potato microplants by acetylsalicylic acid. *Journal of Plant Physiology* 151: 74–78.
- López-Delgado, H., J.F. Dat, C.H. Foyer, and I.M. Scott. 1998. Induction of thermotolerance in potato microplants by acetylsalicylic acid and H<sub>2</sub>O<sub>2</sub>. *Journal of Experimental Botany* 49: 713–720.
- Mahmudi, A., G. Queval, S. Chaouch, S. Vanderauwera, F. Van Breusegem, and G. Noctor. 2010. Catalase function in plants: a focus on arabidopsis mutants as stress-mimic models. *Journal of Experimental Botany* 61: 4197–4220.
- Mora-Herrera, M.E., H. López-Delgado, A. Castillo-Morales, and C.H. Foyer. 2005. Salicylic acid and H<sub>2</sub>O<sub>2</sub> function by independent pathways in the induction of freezing tolerance in potato. *Physiologia Plantarum* 125: 430–440.
- Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.
- Pan, Q., J. Zhan, H. Liu, J. Zhang, J. Chen, P. Wen, and W. Huang. 2006. Salicylic acid synthesized by benzoic acid 2-hydroxylase participates in the development of thermotolerance in pea plants. *Plant Science* 171(2): 226–233.
- Rosenberg, V. 2000. The virus eradication of seed potato initial material. *Plant Breeding and Seed Production* 8: 161–166.
- Sánchez-Rojo, S., H.A. Lopez-Delgado, M.E. Mora-Herrera, H.I. Almeyda-Leon, and H.A. Zavaleta-Mancera. 2011. Salicylic acid protects potato plants from phytoplasma-associated stress and improves tuber photosynthate assimilation. *American Journal of Potato Research* 88: 175–183.
- Senaratna, T., D. Touchell, E. Bunn, and K. Dixon. 2000. Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. *Plant Growth Regulation* 30: 157–161.
- Shi, Q., Z. Bao, Z. Zhujun, Q. Ying, and Q. Qian. 2006. Effects of different treatments of salicylic acid on heat tolerance, chlorophyll fluorescence, and antioxidant enzyme activity in seedlings of *Cucumis sativus* L. *Plant Growth Regulation* 48: 127–135.
- Slezak, I., M. Libik, B. Karpinska, S. Karpinski, and Z. Miszalski. 2007. The role of hydrogen peroxide in regulation of plant metabolism and cellular signaling in response to environmental stresses. *Acta Biochimica Polonica* 54: 39–50.
- Suzuki, N., and R. Mittler. 2006. Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. *Physiologia Plantarum* 126: 45–51.
- Uchida, A., A.T. Jagendorf, T. Hibino, T. Takabe, and T. Takabe. 2002. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. *Plant Science* 163: 512–523.
- Vlot, A.C., D.M.A. Dempsey, and D.F. Klessig. 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology* 47: 177–206.
- Wang, L.J., and S.H. Li. 2006. Salicylic acid induced heat or cold tolerance in relation to Ca<sup>2+</sup> homeostasis and antioxidant systems in young grape plants. *Plant Science* 170: 685–694.
- Wang, Y., J. Zhang, J.L. Li, and X.R. Ma. 2014. Exogenous hydrogen peroxide enhanced the thermotolerance of *Festuca arundinacea* and *Lolium perenne* by increasing the antioxidative capacity. *Acta Physiologiae Plantarum* 36: 2915–2924.
- Warm, E., and G.G. Laties. 1982. Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. *Phytochemistry* 21: 827–831.
- Willekens, H., D. Inze, M. Van Montagu, and W. van Camp. 1995. Catalases in plants. *Molecular Breeding* 1: 207–228.
- Zhou, J., J. Wang, K. Shi, X.J. Xia, Y.H. Zhou, and J.Q. Yu. 2012. Hydrogen peroxide is involved in the cold acclimation-induced chilling tolerance of tomato plants. *Plant Physiology and Biochemistry* 60: 141–149.