INVOLVEMENT OF REACTIVE OXYGEN SPECIES AND THE INDUCTION OF THE CELLULAR ANTIOXIDANT MACHINERY IN THE NECROTIC DEATH OF TWO *CAPSICUM ANNUUM* CULTIVARS WITH DIFFERENT SENSITIVITY TO *PHYTOPHTHORA CAPSICI*

M.E. Requena[1], C. Egea-Gilabert[2] and M.E. Candela[1]

[1]*Biología Vegetal, Facultad de Biología, Universidad de Murcia, Campus de Espinardo, 30100, Espinardo (Murcia), Spain*
[2]*Ciencia y Tecnología Agraria, E.T.S. Ingeniería Agronómica, Universidad Politécnica de Cartagena, C/Paseo Alfonso XIII 48, 30203, Cartagena, Spain*

SUMMARY

This study analyses the defence response of two pepper cultivars, Serrano Criollo de Morelos (SCM, resistant) and California Wonder (CW, sensitive) to oomycete pathogen *Phytophthora capsici.* We compare the production of reactive oxygen species (O_2^- and H_2O_2) *in vitro* and *in vivo*, as well as the activity of antioxidative enzymes in the cuts of the decapitated stems elicited with *P. capsici*.

In all tests, following elicitation O_2^- and H_2O_2 are produced in both cultivars, but in higher amount in SCM cells. *In vivo* assays show that infection by the pathogen causes progressive necrosis in the decapitated SCM and CW stems and that necrosis length is inversely proportional to resistance. The antioxidative enzymes tested (superoxide dismutase, peroxidase, catalase and glutathione reductase) increase their activity after infection, to a significant extent in SCM plants and to a lesser extent in CW plants.

Therefore, we conclude that if H_2O_2 were to play a role in resistance, it would be due to its possible signalling function. These findings are corroborated by the results of our analysis of the spatial distribution of H_2O_2 production along the stem, since in the SCM cultivar H_2O_2 penetrates deeper, and can be detected in regions not reached by the *P. capsici* hyphae. The accumulative effect of oxidative damage mediated by H_2O_2 plus the attainment of homeostasis in SCM plants results in cells being capable of a defense response that is efficient enough to inhibit pathogen growth. Therefore, reactive oxygen species production, lipid peroxidation increase and antioxidative enzymes change play an important role in the resistance of this pepper cultivar to *P. capsici*.

Keywords: hydrogen peroxide, superoxide anion, catalase, peroxidase, lipid peroxidase, superoxide dismutase.

Corresponding author: C. Egea-Gilabert
Fax: +34.968.32.57.93
E-mail: catalina.egea@upct.es

INTRODUCTION

Phytophthora capsici is an oomycete that attacks the pepper plant, causing generalised wilting and plant death a few days after the first symptoms of infection are observed (Candela *et al*., 1995). The disease can only be avoided by using resistant pepper cultivars or by locating the culture in sanitised areas, since soil treatment the highly effective disinfectant methyl bromide is banned.

When it comes to the interaction between *P. capsici* and pepper plants, there has been a good deal of research on the defence response of two plant cultivars with differing sensitivity to the oomycete pathogen, Serrano Criollo de Morelos (SCM, resistant) and California Wonder (CW, sensitive). In the stems of these cultivars inoculated by decapitation with *P. capsici*, various compounds have been found that undergo alterations as a defense response, such as phenolic acids (Candela *et al*., 1995), phytoalexin capsidiol (Egea *et al*., 1996a), proteins with peroxidase activity (Alcazar *et al*., 1995; Egea *et al*., 2001), chitinase (Egea *et al*., 1996b), glucanase and its related genes (Egea *et al*., 1999) and even nitric oxide (Requena *et al*., 2005). All these compounds are involved in one way or the other in processes that inhibit pathogen growth. Up till now, however, no single compound has been identified with a decisive role in the resistance mechanism so as to be deemed responsible for halting the advance of the pathogen.

The defense response of a plant involves numerous reactions, many of which are mediated by the formation of reactive oxygen species (ROS) (Apel and Hirt, 2004). Different ROS may act directly as pathogen killers and/or as intra or extracellular carriers of the defence signal (Mino *et al*., 2004; Torres *et al*., 2006). According to Doke *et al.* (1996), anion superoxide (O_2^-) is initially produced, but due to its high oxidative capacity, it is very toxic for the cell. The latter therefore relies on superoxide dismutase (SOD) to convert it immediately into hydrogen peroxide (H_2O_2), a less reactive compound. Among ROS, H_2O_2 in particular is important in signaling because of its relatively low toxicity, and its ability to cross cellular membranes through aquaporins and thereby migrate to different cellular compartments (Cuypers *et al*., 2016). These findings

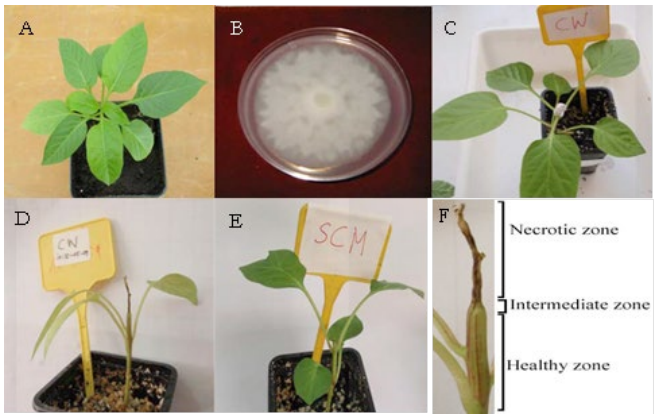

Fig. 1. (A) Healthy 3 month-old pepper plant. (B) *P. capsici* growing on PDA. (C) Pepper plant decapitated and inoculated with the pathogen. (D and E) Pepper plants after 6 days from stem inoculation with the pathogen, showing descending necrosis; (D) CW cultivar, sensitive and (E) SCM cultivar, resistant. (F) Details of a pepper plant stem after 6 days from inoculation by decapitation with *P. capsici*, showing the regions defined for our work in the histochemical ROS study (intermediate region) and antioxidative enzymes (intermediate and healthy).

also show that plants respond to penetration processes in different ways, depending on pathogen activity penetrating or causing only some injury. Specifically, H_2O_2 has been proposed as second messenger for the activation of defense genes in response to injury, and these genes are different from those induced during plant/pathogen interactions (Choi *et al*., 2009). Wounding stimuli also induce H_2O_2 production in both local and systemic tissue (Orozco-Cárdenas *et al*., 2001; Miller *et al*., 2009).

Equilibrium between oxidative damage and the activity of antioxidants determines whether plant cells live or die from HR. ROS are highly toxic compounds for the cell and can oxidise biological macromolecules such as lipids, proteins and nucleic acids, causing lipid peroxidation, damaged membranes and enzyme inactivation. Throughout their evolutionary process, plants have developed antioxidant defence mechanisms to avoid the deleterious effects of ROS. They include certain enzymes – such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX) and glutathione reductase (GR) – as well as non-enzymatic compounds such as ascorbate-glutathione-cycle, whether oxidised (GSSG), reduced (GSH) or total (GLU). SOD converts the O_2^- into H_2O_2, any excess of H_2O_2 being eliminated by peroxidases. POX reduces H_2O_2 to water using various substrates as electron donors. APX uses ascorbate to reduce H_2O_2 to water. CAT can break H_2O_2 into water and oxygen, eliminating the excess of H_2O_2 (Horvath *et al*., 2007). Defense mechanisms against oxidative damage caused by ROS have been observed in many plants infected by pathogen agents. They involve alteration of the levels of ROS produced and those of their antioxidant enzymes (Auh and Murphy, 1995), as well as the activation of protein kinases (Xu *et al*., 2014). According to Bailey-Serres and Mittler (2006), the evolution of efficient mechanisms for the elimination of ROS has enabled plants to overcome their toxicity so as to allow them to function as signalling molecules. Notwithstanding these findings, there is still controversy regarding the response to oxidative stress due to biotic stress, since such response is highly determined by the interaction between specific plants and pathogens. Looking into ROS/enzymatic alterations in interactions between resistant and sensitive plant cultivars may shed light on their role in resistance mechanisms.

This study seeks to investigate the production of O_2^- and H_2O_2 spatially and temporally, by comparison between two pepper varieties, CW and SCM that exhibit different degrees of sensitiveness to *P. capsici*. We also analyse the role of antioxidant enzymes in the defense reactions of each of these two pepper cultivars. Our assays of time-dependent ROS production were based on cellular suspensions of peppers infected at different times with an elicitor, obtained from filtrates of the liquid cultures, of *P. capsici*. For our study of spatial production of ROS, we used pepper plant stems inoculated by decapitation, following both histological and histochemical detection methods. When studying the intracellular H_2O_2 dynamics, we sought to determine the role of H_2O_2 as a signalling molecule linked to resistance in this interaction.

MATERIALS AND METHODS

Plant material. Pepper plants (*Capsicum annuum* L.) cvs. CW (susceptible) and SCM (resistant) were grown from seed in a Fisson climate chamber at 25ºC with 175 µmol m^{-2} s^{-1} photosynthetically active radiation (Osram L 40W/10S lamps) for a 16 h photoperiod and at a relative humidity of 75-80% (Fig. 1A).

Pepper cellular suspensions were obtained according to García-Pérez *et al.* (1998) with certain modifications: seeds of CW and SCM were sterilised by immersion in a calcium hypochlorite solution at 10% for 2 min, followed by 4 washing cycles of 5 min each in sterile distilled water. Seeds were then left to dry in a laminar airflow chamber. Finally, they were placed in Erlenmeyer flasks with MS (4.4 g l^{-1}), 0.8% (w vol^{-1}) agar (Difco) and 3% sucrose, adjusted to pH5.8. Morel vitamins supplements were added with the following composition: 50 ml water, 0.05 g calcium pantothenate, 5 g myo-inositol, 0.05 g nicotinic acid, 0.05 g thiamine, 0.05 g pyridoxine and 0.0005 g biotin. Seeds were left to germinate in a climate chamber at 25ºC with a 16 h photoperiod, until they presented 2 true leaves. At this time, segments of 1 cm were collected from cotyledon explants, and of these, the proximal and distal parts, with the top surface in contact with the medium, were cultured in 50 ml Erlenmeyer flasks containing MS-PDA medium supplemented with 5% sucrose and 1 mg l^{-1} 2,4- dichlorophenoxy-acetic acid (2,4-D). After three weeks the calluses

used to obtain the cellular suspensions were produced. Five g of friable calluses were crumbled in 50 ml of MS medium containing 5% sucrose and 1 mg l^{-1} 2,4- D. This was shaken at 150 rpm for 30 days until they reached a degree of cell packaging of 50-60%. For repetitive assays, the pepper cells had to be sub-cultured every 3 weeks and transferred to fresh MS-PDB medium.

Pathogen and elicitor preparation. The pathogen used was the oomycete *P. capsici* Leonian, isolate 17, which was maintained in the dark on potato dextrose agar (PDA) at 25ºC (Fig. 1B).

To obtain the elicitor, *P. capsici* was grown in Murashige and Skoog medium (MS), supplemented with potato dextrose agar (MS-PDA) at 8% in Petri dishes. From a new 5-day culture, 1 cm^2 discs were taken from the colony's edge, and added to 100 ml flasks containing 20 ml of MS-potato dextrose broth medium (MS-PDB) at 8%. The culture was then placed in an orbital shaker (at 25 rpm) in a culture chamber with continuous lighting at 25ºC. The oomycete grew as a white sphere and released metabolic compounds into the medium. After 30 days, the compounds that forms the "elicitor" were collected (referred to as EF). Immediately prior to use, the EF was filtered through a 0.2 µm, GF/C grade Whatman filtering disk (Maidstone, UK).

Elicitation of pepper cellular suspensions and measurement of O_2^- and H_2O_2. To elicit the pepper cells, 500 µl of SCM or CW cellular suspension at 60% cell packaging were placed in 1.5 ml eppendorf tubes. 500 µl of EF were added to each tube and left to rest for periods of 5, 10, 30 min and 1, 8, 24 h. After each infection period, the tubes were centrifuged at 10,000 rpm for 2 min, after which 500 µl of supernatant were discarded. O_2^- was measured qualitatively by reduction of nitroblue tetrazolium (NBT, Sigma-Aldrich) from a NBT reagent stock. Briefly, 0.5 g NBT (1%) and 0.06501 g of 20 mM NaN_3 were dissolved in 50 ml of 20 mM phosphate buffer, pH 7.5. Staining with NBT was carried out by adding to each eppendorf containing the previous medium 500 µl of NBT reagent. The mix was left to react in the dark at room temperature for 30 min, and then placed in a bath at 85ºC for 15 min to accelerate the reaction. Control tubes contained 500 µl of cellular suspensions and 500 µl of MS-PDB medium without EF.

The detection of H_2O_2 was carried out using 3,3´-diaminobenzidine (DAB, Fluka) according to Thordal-Christense *et al.* (1997). To analyse the H_2O_2 produced by the *in vitro* cells elicited with *P. capsici*, 1 ml of above each cellular suspension of SCM and CW was placed in a well of a multi-well plate, adding 1 ml of EF and leaving it to react during periods of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 minutes, after each of which the reaction was halted by adding 1 ml of DAB solution. Cells were then incubated in the growth chamber for an additional 8 h period to allow DAB uptake and reaction with H_2O_2 and peroxidase. The control test was carried as above but without the addition of EF.

Readings from the cell suspensions were taken at 560 nm in an INFINITE 200 spectrophotometer (OD560 nm) against a blank of medium and DAB. We took the average of 9 spots in each well and worked out from these absorbance values the accumulation of H_2O_2 using a calibration line built as follows: based on mother solution prepared immediately before use of H_2O_2 20 mmol ml^{-1}, dilutions with milli-Q ultrapure water were prepared at concentrations 0.65; 1.25; 2.5; 5.0 and 10.0 mmol ml^{-1}. Absorbance at 560 nm was plotted in the y-axis and H_2O_2 concentration in mmol ml^{-1} was plotted in the x-axis. Accumulation of H_2O_2 measured as staining intensity, using the INFINITE 200 spectrophotometer at 560 nm, is presented in arbitrary units (Vidović *et al.*, 2016).

Assay of the modulation of the system through specific cell protectants. Two compounds were tested to measure how H_2O_2 production is able to change the effects induced by the pathogen, using the method of Frew *et al.* (1983). The compounds (SIGMA) were: a**)** Catalase (CAT) (EC 1.11.1.6 from bovine liver) 500 units ml^{-1} prepared in phosphate buffer 0.05 M and pH=7.0 and b) Superoxide dismutase (SOD) (EC 1,15.1.1) 150 units 10 ml^{-1} prepared in 50% glycerol/10 mM Tris-Mes pH=7.5 buffer. Both compounds were prepared as stock solutions of 100, 50 and 25 µg ml^{-1}, amongst which one (50 µg ml^{-1}) was used in subsequent experiments. 100 µl of each compound solution was later added to 3 ml of cellular pepper suspensions, 30 minutes before the addition of EF which was maintained at 25ºC for 6 h. Control tests were carried out as just described for the samples tests, using the same media, but without addition of EF.

H_2O_2 production in the samples tested and their controls were measured as described in the previous section.

Spatial detection of ROS in pepper stems inoculated with *P. capsici*. Plants (with 5-6 true leaves) of each cultivar, SCM and CW were infected with plugs of actively growing *P. capsici* mycelium (Candela *et al.*, 1995), inoculated by cutting off the tops of the stems (Fig. 1C). Control stems were inoculated using PDA medium alone. Three, six and nine days after inoculation, cylinders of each tissue of necrotic, intermediate (0.5 cm below the necrotic zone), and healthy stems of both cultivars and their controls were collected and further used in the histochemical studies. (Fig. 1). Each assay used 10 plants and was performed at least twice. For each infection period, the necrosis length was measured in each of the 10 plants of the two cultivars, the necrotic region was discarded and the intermediate region is collected, as well as the one we referred to as healthy region, located below the intermediate region and showing no apparent symptoms (Fig. 1F).

Six days after infection by decapitation with *P. capsici*, the detection of ROS was carried out by histochemical

Table 1. Necrosis length and oomycete pathogen penetration (after necrosis zone), along stems of CW (sensitive) and SCM (resistant) pepper cultivars, after 3, 6 and 9 days from inoculation by decapitation with *P. capsici*. Oomycete penetration was analyzed in each 0.5 mm section and measured from the end boundary of the necrotic region, at each tested time lapse. Values are the mean ± standard deviation from 20 plants. Different letters indicate significant differences according to Duncan's test ($P \leq 0.05$).

CW			SCM	
Time after infection (days)	Necrosis length (mm)	Oomycete penetration section and depth (mm)	Necrosis length (mm)	Oomycete penetration section and depth (mm)
3	21.0 + 0.2a	Sec. 29; 14.5 + 0.5	1.7 + 0.4a	Sec. 4; 2 + 0.5
6	56.3 + 0.4b	Sec. 21; 10.5 + 0.5	5.3 + 0.9b	Sec. 2; 1 + 0.5
9	77.0 + 0.3c	Whole stem	5.7 + 0.9b	0 + 0.5

staining (Requena *et al*., 2005). Briefly, the stem cylinders were enclosed in sauco medulla. Using a microtome, semi-fine sections of 200 µm were made serially from top to bottom of the cylinder, starting by the intermediate region (immediately below the necrotic region). The discs were placed in a well plate in the same order as they were made, and were stained to locate *in situ* O_2^- and H_2O_2.

The histochemical detection of O_2^- was performed by infiltrating the stems discs directly with 0.1 mg ml^{-1} NBT staining solution in 25 mmol K-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid] buffer (pH 7.5) and incubating at 24°C in the dark for 1 h. For the control test we used stems discs from non-infected plant, stained in the same way as already described. O_2^- was visualized as a blue/violet colour.

The histochemical detection of H_2O_2 was performed by infiltrating stems discs directly with 0.1 mg ml^{-1} DAB staining solution in 50 mM Tris-acetate buffer (pH 5.1) and incubating at 24°C in the dark for 24 h (Romero-Puertas *et al*., 2004). The discs from non-infected plants were stained as already described. H_2O_2 was visualized as an ochre/brown colour.

The stained stem discs were placed in slides ordered from the start of the intermediate zone, (bordering the necrotic zone) along the stem towards the healthy zone. Stem sections were observed with an Optika B600Ti microscope (Optik, Systeme Gmbh). Each experiment was repeated at least three times with four replicates and a mean of three seedlings stems were analyzed in each replication.

Antioxidant enzyme activity and lipid peroxidation in pepper stems inoculated with *P. capsici*. Measurements of CAT, POX, SOD, APX, GR enzymes and lipid peroxidation (LP) were taken from the intermediate (0.5 cm below necrotic) and healthy (0.5 cm below intermediate) regions in the stems of 20 plants 6 days after infection with *P. capsici*. For the assays the INFINITE 200 spectrophotometer was used, thermostat set at 25°C. SOD activity was determined according to Beauchamp and Fridovich (1971), one SOD activity unit (UA) was defined as the amount of enzyme required to inhibit 50% of NBT photo reduction. POX activity was determined according to Ros-Barcelo (1998) based on the H_2O_2-dependent oxidation of 4-methoxy-α-naphthol (4-MN) at 593 nm. Measurement of APX was based on the ascorbic acid oxidation with H_2O_2 at 290 nm for 1 min (Hossain and Asada, 1984). CAT was assayed measuring decrease of absorbance at 240 nm due to progressive depletion of H_2O_2 (Del Río *et al*., 1977). Measurement of GR (E: 1.6.4.2) was based on the decrease in absorbance at 340 nm due to oxidation of NADH during reduction of GSSG to GSH (Edwards *et al*., 1990). Measurement of LP was carried out by determining the substances that react with thiobarbituric acid (Cakmak and Horst, 1991). All enzymatic activities obtained were expressed as nM or µM (g FW min)$^{-1}$. LP was expressed as nM MDA - malondialdehyde (g FW) $^{-1}$.

Statistical analysis. The experiments were conducted using a completely randomized design. Statistical data analysis was carried out by SPSS package program. The statistical significance between mean values was determined by one-way analysis of variance (ANOVA); Duncan's multiple range test was performed if the differences between the groups were identified as $\alpha = 0.05$.

RESULTS

Evaluation of plant resistance. Necrosis length, depth of pathogen penetration. Fig. 1 shows the plants used (SCM and CW), a Petri dish with growing oomycete in PDA, necrosis in each pepper variety and the stem of a pepper plant after 6 days from inoculation by decapitation with *P. capsici*, where the three zones (necrotic, intermediate and healthy) can be observed. The region defined for our work in the histochemical ROS study is the intermediate region, whereas the intermediate and healthy regions were those analysed for our study of antioxidant enzymes.

The *in vivo* tests performed in pepper stems decapitated and inoculated with *P. capsici* showed that the progressive necrosis induced by the pathogen along the stem are different for each cultivar, being resistance inversely correlated with necrosis length (Table 1). Oomycete hyphae penetration, measured from the necrotic region, reaches after 3 days after inoculation 14.5 mm in the sensitive cultivar CW (corresponding to necrosis of 29 sections of 0.5 mm length). At 6 days, in CW, necrosis had advanced

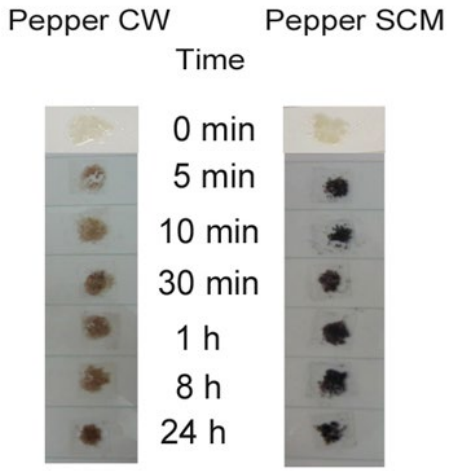

Fig. 2. Nitro blue tetrazolium (NBT) stain for O_2^- production in cellular suspensions of pepper: CW (left) and SCM (right), after different times of elicitation with a *P. capsici* filtrate.

over a further 10.5 mm more (21 sections), measured from the boundary of the necrotic region at 3 days. This makes a total 25 mm from origin. At 9 days, the CW stem was invaded and necrosed to its full length. In contrast, oomycete penetration in the SCM resistant cultivar reached only 2 mm after 3 days (4 sections) and a further 1 mm after 6 days (2 sections). At 9 days after infection, no further necrosis had taken place, meaning that pathogen invasion was halted. In sum, the defense response in the SCM resistant cultivar was able to stop pathogen growth and arrested the infection, whereas in the CW sensitive cultivar, necrosis continued to progress along the stem until it is fully necrosed and the plant dies.

Qualitative analysis of O_2^- in elicited cellular suspensions. *In vitro* assays carried out in pepper cell suspensions, treated with the EF of *P. capsici*, resulted in a HR that translates in necrosis, the latter being more intense in SCM cells than in CW cells. O_2^- was formed instantly with different intensity in both cultivars and did not increase over time (Fig. 2). SCM cells produced much higher amounts of O_2^- than CW cells, from the moment of elicitation and at all periods analysed.

Accumulation of H_2O_2 in elicited cellular suspensions. H_2O_2 was produced in both cultivars instantaneously, but production varied over time (Fig. 3). It increased during the first 3 minutes, at similar levels albeit slightly higher in SCM cells. As of 4 minutes, however, differences became clearly significant between the two cultivars. H_2O_2 accumulation reached 0.5 arbitrary units and did no longer increased in CW cells, whereas it continued to increase in SCM cells, reaching up to 1.4 arbitrary units at 9 min, when production stabilised.

Assay of the modulation of the system through specific cell protectants. When CAT was added, H_2O_2 production decreased significantly in both cultivars (Fig. 4),

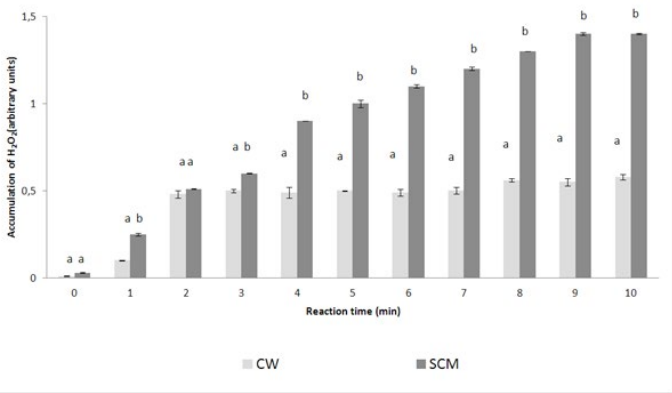

Fig. 3. Time course of H_2O_2 quantification, by 3,3'-diaminobenzidine (DAB) stain measured at 560 nm, in pepper suspension cells, SCM and CW, elicited with *P. capsici* filtrate over time. Values are means ± standard deviations from three independent experiments. Error bars indicate the standard errors of the means. The results were analyzed by Duncan's test following one-way analysis of variance (ANOVA). Values with diverse letters are significantly different ($P \leq 0.05$). Accumulation of H_2O_2, measured as staining intensity is presented in arbitrary units.

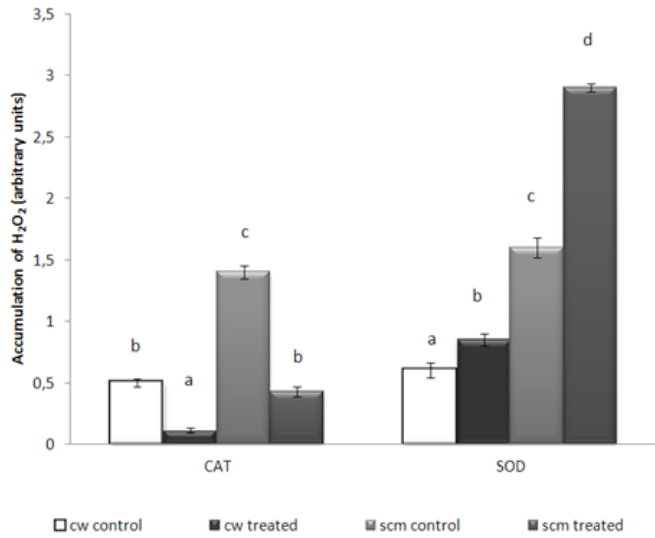

Fig. 4. Effects on H_2O_2 production by adding of CAT and SOD on cells of CW and SCM peppers, incubated with each compound during 30 min and then treated with *P. capsici* filtrate during 6 hours. The control consisted on cells of CW and SCM peppers, incubated with each component dilution medium alone. Values are means ± standard deviations from three independent experiments. Error bars indicate the standard errors of the means. The results were analyzed by a Duncan's test following one-way analysis of variance (ANOVA). Values with diverse letters are significantly different ($P \leq 0.05$). Accumulation of H_2O_2, measured as staining intensity is presented in arbitrary units.

being higher in SCM peppers compared to elicited cells without CAT added. In the case of SOD, addition entailed a significant rise in H_2O_2 in the cells of both cultivars, being the production of H_2O_2 significantly higher in the resistant cultivar than in the susceptible one (Fig. 4).

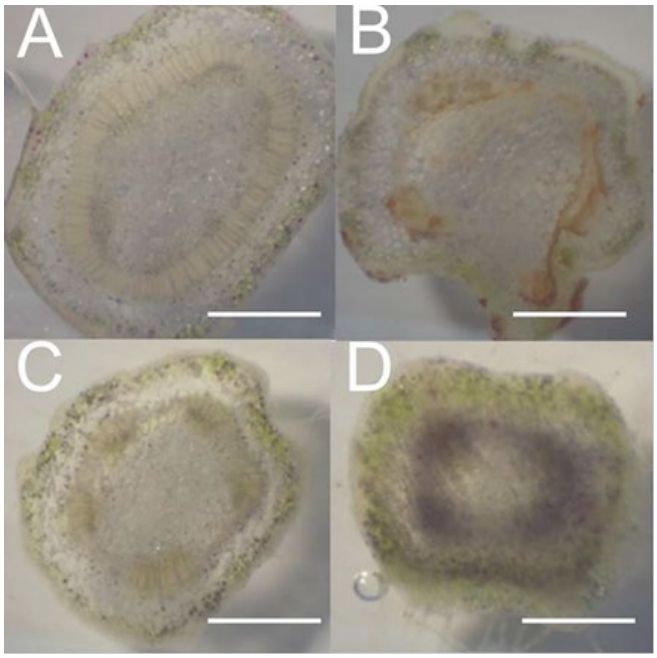

Fig. 5. Nitro blue tetrazolium (NBT) stain for O_2^- production in semi-fine sections from the intermediate region of CW (sensitive) and SCM (resistant) pepper stems. A) Healthy CW stem section. C) Healthy SCM stem section. B) Infected CW stem section and D) Infected SCM stem section, both B and D after 6 days from inoculation with *P. capsici*. The bars represent 500 µm.

Spatial detection of ROS in pepper stems inoculated with *P. capsici*. O_2^- was produced following an abiotic stress (tissue injury on cutting) and even more following biotic stress (infection), in the two cultivars (Fig. 5). The production of O_2^- was slightly more intense in SCM healthy (control) plants (Fig. 5C) than in CW healthy (control) plants (Fig. 5A), possibly due to the tissue damage caused by cutting. However, in infected stems, NBT stained vascular bundle cells was more intense in SCM plants (Fig. 5D), where O_2^- concentrates, in both in phloem and xylem vessels than in CW infected plants (Fig. 5B), where there is also O_2^- production in the vascular system, but less intense than in SCM.

H_2O_2 production was measured spatially along the stem in semi fine sections (200 µm) after inoculation with *P. capsici* for 6 days. Figs. 6, 7 and 8 show 10 sections from CW and SCM plants stained with DAB, respectively. It can be seen that after the infection, H_2O_2 is produced by both cultivars in vascular bundles as in epidermic cells (Fig. 6; Fig. 7; Fig. 8). Also, production concentrates essentially in the vascular system. Sections from the sensitive CW cultivar in Fig. 6 show H_2O_2 production (as staining intensity) is more or less uniform from section 1 to 6. A more intensely stained zone can be observed, which could correspond to the phloem, and although all the vascular system appears stained, xylem vessels, by their lighter staining, appear to contain less H_2O_2. As of section 7, the staining intensity of the whole vascular system is visibly

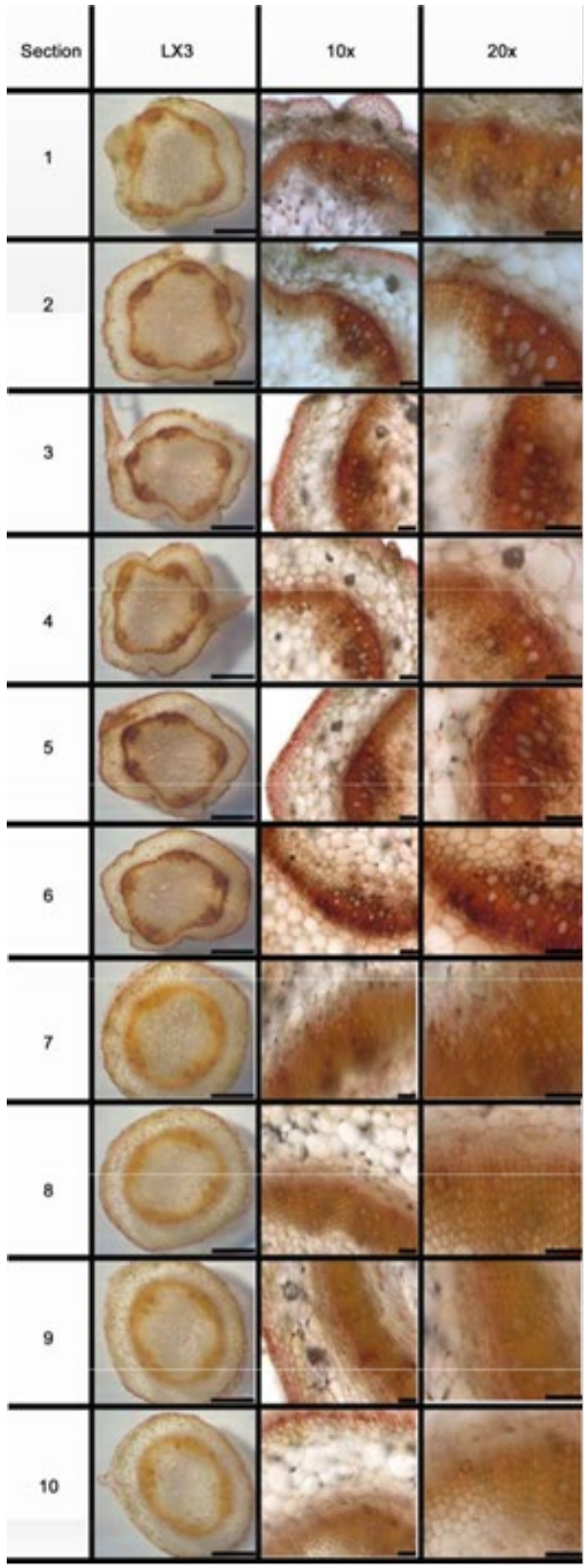

Fig. 6. Detection of H_2O_2 in CW pepper stems decapitated and inoculated with *P. capsici* mycelia disks for 6 days. 10 sequential sections (200 µm) from the intermediate region (immediately below the necrotic region) are shown, stained with DAB for 1 h. The bars represent 300 µm in the first column (LX3), 20 µm in the second column (10×) and 50 µm in the third column (20×).

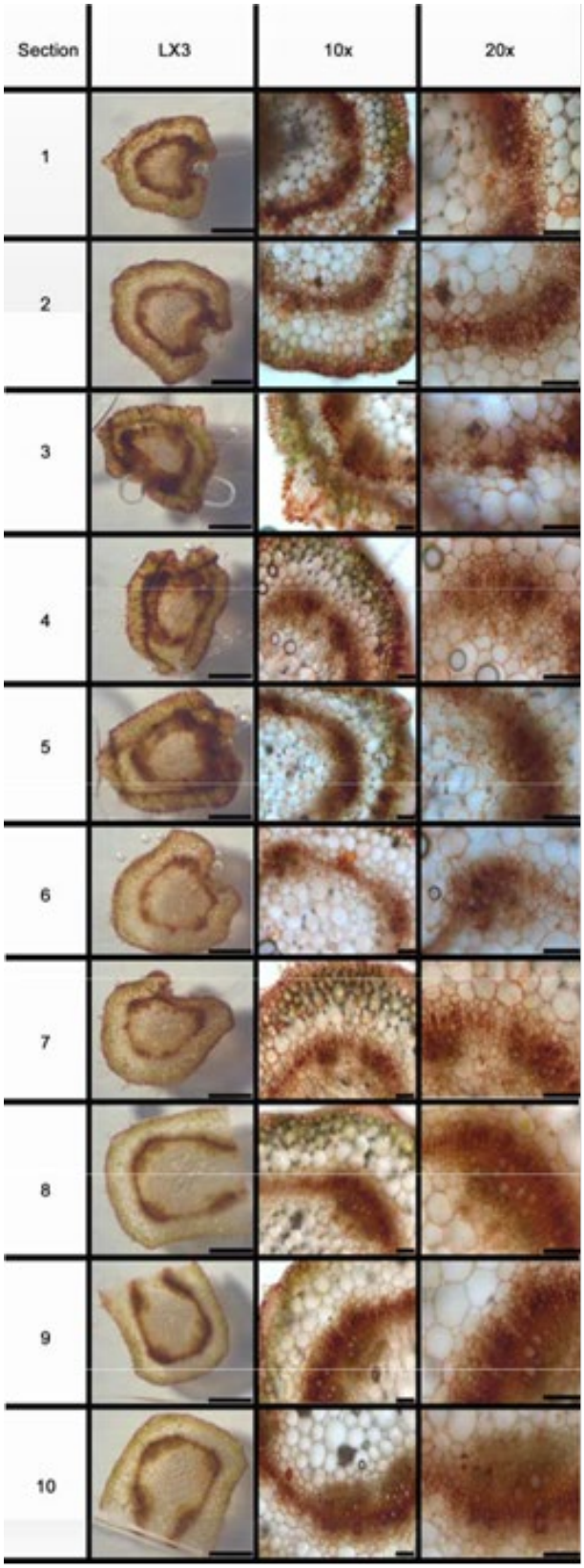

Fig. 7. Detection of H_2O_2 in SCM pepper stems decapitated and inoculated with *P. capsici* mycelia disks for 6 days. 10 sequential sections (200 µm) from the intermediate region (immediately below the necrotic region) are shown, stained with DAB for 1 h. The bars represent 250 µm in the first column (LX3), 25 µm in the second column (10×) and 50 µm in the third column (20×).

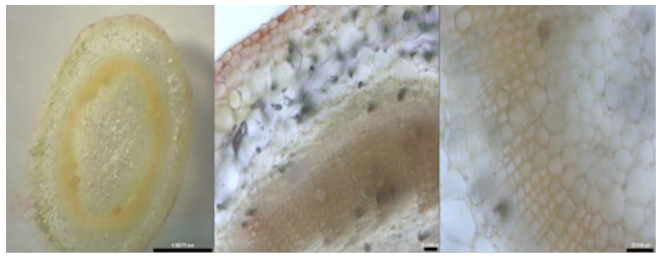

Fig. 8. Production control of H_2O_2 in pepper stems, decapitated and inoculated with PDA medium disks without *P. capsici*. One CW stem section of 200 µm thin from the intermediate region stained with DAB for 1 h, is shown. The bars represent 500 µm in the first column (LX3), 25 µm in the second column (10×) and 25 µm in the third column (20×).

lower, almost fading out by sections 9 and 10. In the epidermis, production is visible in the first sections, but as of section 8 several epidermic cells are visibly free of stain. If we correlate H_2O_2 production with signalling, we observe that detection weakens in CW plants as of section 7 (1.4 mm). Therefore, defence response signals reach less depth and are of lower intensity in the sensitive cultivar. Sections from resistant SCM plants (Fig. 7) show H_2O_2 production levels both in the epidermic and in the vascular system that are much higher than those seen in CW plants. The most significant observation is that staining is intense up to section 10 (the last analysed), without fading after section 7 as seen in the CW cultivar. In SCM plants, H_2O_2 production reaches further along the stem (2.0 mm) and when set in relation to signalling, H_2O_2 informs more cells under attack by the pathogen, thereby inducing a more intense and far-reaching defence response. Fig. 8 shows sections of stem tissue from CW not elicited with *P. capsici,* corresponding to controls. No difference can be observed between CW and SCM tissue (data not shown).

Antioxidant enzymatic activity and lipid peroxidation in pepper stems inoculated with *P. capsici*. Antioxidant enzyme activity (SOD, POX, GR and CAT) was higher in the SCM resistant cultivar in the 2 stem zones analysed (intermediate and healthy zones, Fig. 1F), compared to the CW sensitive one (Table 2). The most significant increase in SOD enzyme activity took place in the intermediate region of the resistant cultivar, where the increase rate [(Intermediate-Healthy)/Healthy)×100)] reached 154.7% compared to the levels seen in the CW that only increased 75.6%. POX activity was similar in the healthy region (used as control) in both cultivars, whereas in the intermediate region, the increase rate was 126.5% in SCM and 102.2% in CW plants. On the contrary, APX activity increased significantly in the intermediate region compared to the healthy region, at an increase rate of 177.2% in CW and 71.41% in SCM plants. GR variations were not significant between the two cultivars, although the trend was still observed whereby activity increased in the intermediate region in both cultivars, in contrast with the

Table 2. Superoxide dismutase (SOD), Peroxidase (POX), Ascorbate peroxidase (APX), Glutathione reductase (GR), Catalase (CAT) and Lipid Peroxidation (LP) in each pepper cultivar (CW or SCM) for each analyzed step region (I: intermediate and H: healthy), following 6 days after decapitation and inoculation with *P. capsici.* FW: fresh weight. MDA: malondialdehyde. Values are means ± standard deviations from three independent experiments. The data shown are the average values (n=20) analyzed by Duncan´s test following one-way analysis of variance (ANOVA). Values with diverse letters are significantly different ($P \leq 0.05$).

	SOD nM (g FW min)$^{-1}$	**POX** µM (g FW min)$^{-1}$	**APX** nM (g FW min)$^{-1}$	**GR** nM (g FW min)$^{-1}$	**CAT** µM (g FW min)$^{-1}$	**LP** nM·MDA (g FW)$^{-1}$
SCM						
I	469.5 ± 11b	5257.3 ± 14b	3287.3 ± 14b	61.8 ± 3b	126.5 ± 7b	16.3 ± 1b
H	184.3 ± 9a	2321.1 ± 6a	1917.5 ± 8a	47.5 ± 2a	71.7 ± 4a	7.1 ± 1a
CW						
I	109.6 ± 7b	4244.4 ± 11b	2619.1 ± 9b	49.6 ± 3b	73.2 ± 3b	8.4 ± 1a
H	62.4 ± 5a	2098.6 ± 9a	944.7 ± 5a	38.5 ± 3a	59.9 ± 2a	6.2 ± 1a

results obtained from the healthy region, the increase rate was 30.1% in SCM, slightly higher than in CW (28.8%). Regarding to CAT activity the increase rate was 76.4% in SCM and 22.2% in CW plants. LP levels were higher in resistant plants compared to sensitive ones (Table 2). Peroxidation levels were similar for both cultivars in the healthy region, but increased significantly with the infection (129.6%) in intermediate region of SCM plants, compared to an increase rate of 35.5% in CW plants.

DISCUSSION

By analysing the activity of the oxidative burst components (ROS and antioxidative enzymes) in the interaction pepper plants and *P. capsici* we conclude that resistance is inversely proportional to the necrosis length zone of the stems (Table 1). This indicates that the defense response in SCM plants has been efficient enough to inhibit oomycete growth and repeal the infective invasion, contrary to what is seen in the sensitive variety CW. O_2^- analysis shows that its production occurs immediately after fungal penetration both in sensitive CW cells as in resistant SCM cells, but more intense in the latter (Fig. 2), results that are consistent with those obtained for superoxide anion by Doke *et al.* (1996). The HR that is observed upon elicitation with *P. capsici* of these pepper cultivars is similar to that observed in other pepper cultivars, and the reaction intensity is consistently higher in the resistant cultivars (García-Pérez *et al.*, 1998). In this interaction, the immediate detection of O_2^- in infected tissue, along with the high toxicity of this molecule, suggest that it could be one of the inducers of the HR in the initially infected cells. By inducing necrosis, it could also be deemed as one of the responsible factors of pathogen growth arrest (Van Breusegem and Dat, 2006). However, O_2^- transforms into H_2O_2 very rapidly. Thus, in order to ascertain its role in ulterior defence mechanisms–involving signalling in neighbouring cells–focusing on H_2O_2 is appropriate, as this is the reactive species that persists over time. H_2O_2 forms initially at the same level in cells of both cultivars, but it stabilises relatively soon in CW cells compared with SCM cells, where it continues to increase until it stabilises at a maximum of 1.4 arbitrary units (Fig. 3).

If we identify necrosis as the defence reaction that the plant opposes to pathogen progress *in vivo*, we see that it occurs in both cultivars. However, whereas the reaction is sufficient to arrest the pathogen in SCM plants, the defence compounds produced by CW plants are not sufficient to inhibit pathogen growth, thereby allowing it to continue its progress until it invades the stem fully and kills the plant (Table 1). Among other factors, it is worth noting that in the CW cultivar the pathogen is always seen progressing beyond the necrotic reaction (Table 1). The plant is thus producing defence compounds at the rear of the hyphae progress, where they do not hinder growth. Instead, the hypersensitive reaction plus the availability of defense substances in the resistant cultivar are able to arrest pathogen growth because after 9 days from infection, oomycete growth is no longer observed beyond the necrosis boundary, and this allows the SCM plant to survive. This explanation coincides with that offered by other authors who relate resistance or susceptibility to attack by a pathogen to the concentration of defense substances produced by the plant in the course of the hypersensitive response, and to the rapidity of production (Delledonne *et al.*, 2002).

Our interpretation of the results obtained after adding compound involved in H_2O_2 production (Fig. 4) would be consistent with that offered by Lherminier *et al.* (2009); they assert that H_2O_2 production does not seem determinant in relation to HR triggering, but may be relevant in relation to the signalling pathway associated with plant cell protection. According to Neill *et al.* (2002), H_2O_2 may act as a second messenger activating expression of defense genes not only against pathogens but also in case of injury, thereby acting as a versatile molecule of the ROS network (Quan *et al.*, 2008; Keshavarz-Tohid *et al.*, 2016). The results of the present study, lead us to suggest that reactions leading to an increase in ROS take place both in the vascular tissue and in the epidermis. They can be consistent with those of other authors that link H_2O_2 to systemic signalling (Orczyk *et al.*, 2010). Our results show that there is a difference in *P. capsici* hyphae penetration

and in H_2O_2 detection in stems of each cultivar, whereas in both of them, ROS signalling not only diffuses through the phloem as observed by Ryals *et al.* (1996), but may also be channelled through the xylem (Fig. 6; Fig. 7), as observed by Ros-Barcelo (1998).

The physiological relevance of the signals carried by ROS can be appreciated by the immediate alteration of antioxidative enzymes and the lipid peroxidation that is triggered in both cultivars and in different regions of the stem. In the SCM resistant cultivar, the higher activity of the SOD, POX, APX, GR and CAT enzymes is found in the intermediate region (Table 2), indicative that tissues neighbouring the infected region have triggered defense responses based on the production of antioxidative enzymes. In turn, this indicates that the responses induced by the pathogen are the result of reactions not only in the cells subject to HR, but also in neighbouring tissue which has been informed by signals originating in such cells (Zhang and Tang, 2012).

In this study, the enzymes most related with H_2O_2 (Table 2), namely SOD (production) and CAT (destruction) exhibit a behaviour that reflects the spatial distribution of H_2O_2 in stems inoculated with the pathogen. SOD activity increase in the intermediate regions of both cultivars suggests that SOD protects cells from damages caused by the O_2^-, in a higher proportion in SCM plants than in CW plants (Table 2). The production of H_2O_2 leads to an increase in tissue lignifications, ensuring better protection against pathogens (Gill *et al*., 2010).

It is known that CAT, APX and POX remove H_2O_2 excess in severe stress conditions (Mittler, 2002). Accordingly, an increase in those activities, as seen in our study, could be due to the plant's need to tackle the high H_2O_2 build-up resulting from SOD activity on O_2^- in the intermediate region of the SCM resistant cultivar. The fact that in CW plants there is only a less variation in CAT and POX activity between the healthy and the intermediate regions (increase rate by 22.2% in CAT and 102.2% in POX) indicates that CAT activity is of low significance in terms of the antioxidative response in this cultivar (Table 2). These results coincide with those of Mittler *et al*. (1999), according to which tobacco plants with reduced capability to detoxify reactive oxygen intermediates are more sensitive to pathogen infection.

Another approach to the analysis of the oxidative burst and the plant's reaction to mitigate oxidative stress is based on the glutathione cycle. The results of GR assay (Table 2) indicate that it increases in concentration in both cultivars after infection, but that differences are not significant. We deduce therefore that the reactions in which this enzyme is involved have a lesser relevance in relation to the resistance to the pathogen exhibited by SCM plants, compared to the sensitivity exhibited by the CW cultivar. LP is an indicator of oxidative tissue damage in response to biotic or abiotic stresses (Apel and Hirt, 2004). Its intensity is determined by the pathogen-host relationship. High ROS and free radical production levels may affect the plasma membrane, leading to the loss of cellular integrity and necrosis, in a reaction that the plant triggers to prevent pathogen growth and thereby stop infection. In our study, the high levels of LP observed in the intermediate region of SCM (129.6%) indicate a higher damage in the resistant cultivar tissue than in the susceptible (35.5%) (Table 2), due possibly to a higher production of ROS in SCM. If we look at the intermediate region levels, the increase seen in SCM plants may be indicative of the damage inflicted to cell membranes. Such damage rendered cells unsuitable for pathogen growth, thereby arresting infection. Our results systematically yield higher LP values in the resistant cultivar compared to the sensitive one, consistent with those obtained by Muckenschnabel *et al*. (2002), leading us to confirm that LP may be one of the defence factors at play.

In conclusion, in this study we have confirmed that following elicitation of resistant and sensitive pepper cells with *P. capsici* filtrates, an instantaneous oxidative burst has been visualized with the production of O_2^-, more intensely, in qualitative terms, in the resistant cultivar SCM. Therefore, we relate the oxidative action of O_2^- with the defense reaction of pepper cells, which include the HR. This is essentially because in CW plants it does not increase with time after elicitation (Fig. 2), and in addition this cultivar is not able to stop the pathogen's advance (Table 1). With the potential involvement of SOD, H_2O_2 is produced, reaching higher levels in the SCM cultivar, with concentration increasing over time. Although, SOD increased the level of H_2O_2 in CW, the levels are not enough in this cv. to inhibit the growth of *P. capsici* hyphae. Therefore, we conclude that if H_2O_2 were to play a role in resistance, it would not be due to a direct cytotoxic action on the pathogen hyphae, but rather to its possible signalling function, consistent with the results obtained by Miguel *et al*. (2000). These findings are corroborated by the results of our analysis of the spatial distribution of H_2O_2 production along the stem, since in the SCM cultivar H_2O_2 penetrates deeper, and can be detected in regions not reached by the hyphae, where it likely signals the pathogen invasion to healthy cells so that these trigger the production of defense compounds capable of arresting pathogen growth, This, together with a higher antioxidative enzyme activities in SCM cells allowing homeostasis, suggests that the two processes are important components of the resistance exhibited by the SCM cultivar to infection by *P. capsici,* compared to that exhibited by CW pepper plants.

REFERENCES

Alcazar M.D., Egea C., Espín A., Candela M.E., 1995. Peroxidase isoenzymes in the defense response of *Capsicum annuum* to *Phytophthora capsici. Physiologia Plantarum* **94**: 736-774.

Apel K., Hirt H., 2004. Reactive oxygen species: metabolism,

oxidative stress, and signal transduction. *Annual Review of Plant Biology* **55**: 373-399.

Auh C.K., Murphy T.M., 1995. Plasma membrane redox enzyme is involved in the synthesis of O_2^- and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. *Plant Physiology* **107**: 1241-1247.

Bailey-Serres J., Mittler R., 2006. The roles of reactive oxygen species in plant cells. *Plant Physiology* **141**: 311.

Beauchamp C., Fridovich I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**: 276-287.

Cakmak I., Horst W.J., 1991. Effect of aluminum on lipid-peroxidation, superoxide-dismutase, catalase, and peroxidase-activities in root-tips of soybean (*Glycine*-max). *Physiologia Plantarum* **83**: 463-468.

Candela M.E., Alcázar M.D., Espín A., Egea C., Almela L., 1995. Soluble phenolic acids in *Capsicum annuum* stems infected with *Phytophthora capsici*. *Plant Pathology* **89**: 308-320.

Choi H.W., Lee D.H., Hwang B.K., 2009. The pepper calmodulin gene CaCaM1 is involved in reactive oxygen species and nitric oxide generation required for cell death and the defense response. *Molecular Plant-Microbe Interactions* **11**: 1389-1400.

Cuypers A., Hendrix S., Amaral Dos Reis R., De Smet S., Deckers J., Gielen H., Jozefczak M., Loix C., Vercampt H., Vangronsveld J., Keunen E., 2016. Hydrogen peroxide, signaling in disguise during metal phytotoxicity. *Plant Science* **7**, article 470: 1-25.

Del Rio L.A., Ortega M.G., Lopez A.L., Gorge J.L., 1977. A more sensitive modification of the catalase assay with the clark oxygen electrode. Application to the kinetic study of the pea leaf enzyme. *Analytical Biochemistry* **80**: 409-415.

Delledonne M., Murgia I., Ederle D., Sbicego P.F., Biondian A., Polverari A., Lamb C., 2002. Reactive oxygen intermediates modulate nitric oxide signaling in the plant hypersensitive disease-resistance response. *Plant Physiology and Biochemistry* **40**: 605-610.

Doke N., Miura Y., Sanchez L.M., Park H.J., Noritake T., Yoshiota H., Kawakita K., 1996. The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defence-a review. *Gene* **179**: 45-51.

Edwards E.A., Rawsthorne S., Mullineaux P.M., 1990. Subcellular-distribution of multiple forms of glutathione-reductase in leaves of pea (*Pisum-sativum*-l). *Planta* **180**: 278-284.

Egea C., Alcázar M.D., Candela M.E., 1996a. Capsidiol: its role in the resistance of *Capsicum annuum* to *Phytophthora capsici. Physiologia Plantarum* **98**: 737-742.

Egea C., Alcázar M.D., Candela M.E., 1996b. b-1,3-glucanase and chitinase as pathogenesis-related proteins in the defense reaction of two *Capsicum annuum* cultivars infected with cucumber mosaic virus. *Biologia Plantarum* **38**: 437-433.

Egea C., Dickinson M.J., Candela M., Candela M.E., 1999. β-1,3-Glucanase isoenzymes and genes in resistance and susceptible pepper (*Capsicum annuum*) cultivars infected with *Phytophthora capsici*. *Physiologia Plantarum* **107**: 312-318.

Egea C., Sid Ahmed A., Candela M., Candela M.E., 2001. Elicitation of peroxidase activity and lignin biosynthesis in pepper suspension cells by *Phytophthora capsici*. *Journal of Plant Physiology* **158**: 151-158.

Frew J.E., Jones P., Scholes G., 1983. Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solutions. *Analytica Chimica Acta* **155**: 139-150.

Garcia-Pérez M.D., Egea C., Candela M.E., 1998. Defence response of pepper (*Capsicum annum*) suspension cells to *Phytophthora capsici. Physiologia Plantarum* **103**: 527-533.

Gill T., Sreenivasulu Y., Kumar S., Aingh Ahuja P., 2010. Overexpression of superoxide dismutase exhibits lignification of vascular structures in *Arabidopsis thaliana. Journal of Plant Physiology* **167**: 757-760.

Horvath E., Szalai G., Janda T., 2007. Induction of abiotic stress tolerance by salicylic acid signaling. *Journal of Plant Growth Regulation* **26**: 290-300.

Hossain M.A., Asada K., 1984. Inactivation of ascorbate peroxidase in spinach-chloroplasts on dark addition of hydrogen-peroxide - its protection by ascorbate. *Plant Cell Physiology* **25**: 1285-1295.

Keshavarz-Tohid V., Taheri P., Taghavi S.M., Tarighi S., 2016. The role of nitric oxide in basal and induced resistance in relation with hydrogen peroxide and antioxidant enzymes. *Journal of Plant Physiology* **199**: 29-38.

Lherminier J., Elmayan T., Fromentin J., Elaraqui K.T., Vesa S., Morel J., Verrier J.L., Cailleteau B., Blein J.P., Simon-Plas F., 2009. NADPH oxidase-mediated reactive oxygen species production: subcellular localization and reassessment of its role in plant defense. *Molecular Plant-Microbe Interactions* **22**: 868-881.

Miguel E., Poza-Carrion C., Lopez-Solanilla E., Aguilar I., Llama-Palacios A., Garcia-Olmedo F., Rodriguez-Palenzuela P., 2000. Evidence against a direct antimicrobial role of H_2O_2 in the infection of plants by *Erwinia chrysanthemi*. *Molecular Plant-Microbe Interactions* **13**: 421-429.

Mino M., Abe S., Suzuki T., Yokoyama H., Kaminaka H., Ogawa K., Morita S., Masumura T., Tanaka K., Inoue M., 2004. Hydrogen peroxide functions as a cell death signal for hybrid lethaly in the F1 of *Nicotiana gossei*×*N. tabacum*. *Plant Science* **167**: 267-274.

Miller G., Schlauch K., Tam R., Cortes D., Torres M.A., Schulaev V., Dangl J.L., Mittler R., 2009. The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Science Signalling* **2** (84) ra 45.

Mittler R., Hallak Herr E., Larus Orvar B., Van Camp W., Willekens H., Inzé D., Ellis B.E., 1999. Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. *Proceedings of the National Academy of Sciences USA* **96**: 14165-14170.

Mittler R., 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Science* **7**: 405-410.

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

Neill S.J., Desikan R., Hancock J.T., 2002. Hydrogen peroxide signalling. *Current Opinion in Plant Biology* **5**: 388-395.

Orozco-Cardenas M.L., Narvaez-Vasquez J., Ryan C.A., 2001. Hydrogen peroxide acts as a second messenger for the

induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* **13**: 179-191.

Orczyk W., Dmochowska-Boguta M., Czembor H.J., Nadolska-Orczyk A., 2010. Spatiotemporal patterns of oxidative burst and micronecrosis in resistance of wheat to brown rust infection. *Plant Pathology* **59**: 567-575.

Quan L.J., Zhang B., Shi W.W., Li H.Y., 2008. Hydrogen peroxide in plants: A versatile molecule of the reactive oxygen species network. *Journal of Integrative Plant Biology* **50**: 2-18.

Requena M.E., Egea-Gilabert C., Candela M.E., 2005. Nitric oxide generation during the interaction with *Phytophthora capsici* of two *Capsicum annuum* cultivars showing different degrees of sensitivity. *Physiologia Plantarum* **124**: 50-60.

Romero-Puertas M.C., Rodríguez-Serrano M., Corpas F.J., Gómez M., Del Río L.A., Sandalio L.M., 2004. Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant, Cell and Environment* **27**: 1122-1134.

Ros Barceló A., 1998. Hydrogen peroxide production is a general property of the lignifying xylem from vascular plants. *Annals of Botany* **82**: 97-103.

Ryals J.A., Neuenschwander U.H., Willits M.G., Molina A., Steiner H.Y., Hunt M.D., 1996. Systemic Acquired Resistance. *Plant Cell* **8**: 1809-1819.

Thordal-Christensen H., Zhang Z., Wei Y., Collinge D.B., 1997. Subcellular localization of H_2O_2 in plants, H_2O_2 accumulation in papillae and hypersensitive response during barley-powdery mildew interaction. *The Plant Journal* **11**: 1187-1194.

Torres M.A., Jones J.D.J., Dangl J.L., 2006. Reactive oxygen species signalling in response to pathogens. *Plant Physiology* **141**: 373-378.

Van Breusegem F., Dat J.F., 2006. Reactive oxygen species in plant cell death. *Plant Physiology* **141**: 384-390.

Vidovic M., Morina F., Prokic L., Milic-Komic S., Zivanovic B., Jovanovic S.V., 2016. Antioxidative response in variegated *Pelargonium zonale* leaves and generation of extracellular H_2O_2 in (peri)vascular tissue induced by sunlight and paraquat. *Journal of Plant Physiology* **206**: 25-39.

Xu J., Yang K.Y., Yoo S.J., Liu Y., Ren D., Zhang S., 2014. Reactive oxygen species in signalling the transcriptional activation of *WIPK* expression in tobacco. *Plant, Cell and Environment* **37**: 1614-1625.

Zhang R.Q., Tang M., 2012. Role of hydrogen peroxide and antioxidative enzymes in *Pinus tabulaeformis* seedlings inoculated with *Amanita vaginata* and/or *Rhizoctonia solani*. *European Journal of Plant Pathology* **134**: 381-389.

Received October 15, 2016
Accepted January 19, 2017