

# Effect of Methyl Jasmonate on antioxidative enzyme activities and on the contents of ROS and H<sub>2</sub>O<sub>2</sub> in *Ricinus communis* leaves

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## ABSTRACT

Jasmonates are a class of plant hormones that mediate various aspects in gene and metabolic regulation, defense, stress responses, reproduction and, possibly, communication. Oxidative stress stimulates synthesis of antioxidant metabolites and enhances antioxidant enzyme activities that could protect plant tissues. The aim of this study was to verify the effects of methyl jasmonate (JAME) treatment on the reactive oxygen species (ROS) and on the activities of H<sub>2</sub>O<sub>2</sub> scavenging enzymes, such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX EC; 1.11.1.1), and guaiacol peroxidase (GPX; EC 1.11.1.7) in *Ricinus communis* leaves. The activity of CAT and GPX was transient while SOD activity decreased and APX increased after treatment with JAME. In addition, JAME exposure induced ROS accumulation.

**Key words:** Methyl jasmonate, oxidative stress, antioxidant enzymatic defenses, catalase, superoxide dismutase, peroxidase, *Ricinus comunnis*, signal transduction

**Abbreviations:** Prx, peroxidase (EC 1.11.1.7); APX, ascorbate peroxidase (EC; 1.11.1.1); SOD, superoxide dismutase (EC 1.15.1.1); CAT, catalase (EC 1.11.1.6); AJ, jasmonic acid; JAME, methyl jasmonate; ROS, reactive oxygen species.

## INTRODUCTION

Plants, similar to animals, face many problems: a need for regulation of metabolic processes and reproduction and efficient defense against enemies. They respond to a variety of

environmental stresses (such as high and low temperatures, drought, salinity, UV or ozone stress and pathogen infections) through the induction of antioxidant defense enzymes that provide protection against further damage (Mittler et al., 2004; Hossain et al., 2009; Tanou et al., 2009; Ma and Gao, 2010).

Jasmonic Acid (JA) and its methyl ester (JAME) are linoleic acid-derived cyclopentanone based compounds and key molecules of the octadecanoid signaling pathway. Jasmonates are one of the simplest non-traditional plant hormones with diverse roles and functions, including a potential role in plant defense as part of the complex signaling pathways. JAME does not only regulate a variety of plant-developmental responses, but is also induced by pathogen attack or wounding, which often leads to the generation of reactive oxygen species (ROS), including hydrogen peroxide ( $H_2O_2$ ), superoxide anions ( $O_2^-$ ) and hydroxyl free radicals ( $\bullet OH$ ) (Faurie et al., 2009; Parra-Lobato et al., 2009). ROS have the potential to interact with many cellular components, triggering stresses in plant cell culture, leading to membrane damage and, as a result, there is an immediate cellular response to trigger plant defense signals. Plants possess antioxidant defense systems, consisting of enzymatic and non-enzymatic components, which normally maintain ROS balance within the cell. For instance, they use a diverse array of enzymes, as well as low molecular weight antioxidants such as ascorbate and glutathione (GSH) to scavenge different types of ROS (Mittler, 2002; Jubany-Mari et al., 2010).

Anti-oxidative enzymes include superoxide dismutase (SOD), which catalyzes the disproportion of superoxide radicals to hydrogen peroxide and dioxygen; catalase (CAT) and peroxidase (Prx), which remove  $H_2O_2$ . SOD are metalloenzymes with three known classes, depending on the active site metal cofactor (Mn, Fe, or Cu/Zn), and which play a key role in protection against oxidative stress. Catalases are tetrameric homoproteins that exist as multiple isozymes encoded by nuclear genes. They are located mostly in peroxisomes and glyoxisomes.

Unlike peroxidase, catalase efficiently scavenges  $H_2O_2$  and does not require a reducing substrate to perform the task. Catalase isozymes differ in biochemical properties, as well as in their developmental specificity (occasionally related to germination), but their principal function probably involves fatty acid conversion, whilst other isozymes are related to lignification, photorespiration or the aging process (Kumari et al., 2006). The peroxidases (Prx) are associated with biochemical and physiological processes such as growth, cell formation, fruit development, ethylene biosynthesis, as well as the response to various stresses (Matamoros et al., 2003). Prx are homoproteins of approximately 50 kDa that are present as multiple isozymes in plant tissues; several Prx are present in nodules, but their functions and subcellular localizations are unknown (Becana et al., 1989). APX is the most important

peroxidase in  $H_2O_2$  detoxification, catalyzing the reduction of  $H_2O_2$  to water using the reducing power of ascorbate (Noctor and Foyer, 1998). This enzyme may account for up to 1% of the total soluble protein of nodules, and exists in the parenchyma and infected zone of several determinate nodules such as alfalfa (Dalton et al., 1998). Plants constantly sense and assess the level of ROS and reprogram their gene expression to respond to the changing conditions in their environment. Many studies employing mutants and antisense lines for catalase (CAT) 1 and 2, ascorbate peroxidase (APX) 1 and CuZn-superoxide dismutase 2 (CSD2) have revealed a strong link between ROS and process such as growth, development and biotic and abiotic stress responses. The function of ROS as signaling molecules involved in the expression of a large number of genes was studied using microarray experiments conducted on loss-of-function mutants that lack important ROS-scavenging enzymes. The use of mutants that lack important ROS-scavenging enzymes such as APX1 and CAT enabled therefore the identification of key regulatory proteins involved in the sensing of ROS and the regulation of transcript expression by different ROS signals (Miller et al., 2008).

The aim of the present study was to examine the relationship between reactive oxygen species accumulation and anti-oxidative enzyme activities in *Ricinus communis* plant leaves after treatment with methyl jasmonate for different time durations.

## MATERIAL AND METHODS

Chemicals used for the protease assay were from Sigma. Electrophoresis reagents used were from Bio-Rad. Methyl jasmonate was purchased from Aldrich and Triton X-100 was from Calbiochem.

**Plant material and protein extraction:** Castor bean (*Ricinus communis* L., cultivar IAC-80, from Instituto Agronômico de Campinas, São Paulo, Brazil) seeds were germinated in plastic pots containing a mixture of soil and vermiculite (3:1, v/v) under controlled conditions in a greenhouse. Plants at the three-leaf-stage (15 days) were sprayed with a solution of methyl jasmonate (125 ml in 500 ml distilled water containing 0.125% Triton X-100) (Pearce et al., 2001). Control plants did not receive any treatment. For all treatments, plants were maintained in environmental chambers under constant light ( $200 \mu mol m^{-2} s^{-1}$ ) for 14 h at 28 °C and for 12 h in the dark at 18 °C. At specific intervals after

methyl jasmonate treatment (1, 4, 6, 12, 24 and 48 hours), fresh leaves (3 g) were harvested directly into liquid nitrogen in a mortar, with 0.3 g PVP, in 100 mM potassium phosphate buffer pH 7.5 with 1 mM EDTA and 3 mM DDT.

The homogenate was centrifuged in a refrigerated centrifuge at 15 000 *g* for 20 min, and the supernatant obtained was used as the source of enzyme. Protein concentrations were estimated according to Bradford's method (1976) using bovine serum albumin as standard

**Superoxide Dismutase Activity:** A non-denaturing gel (native PAGE -10%) was carried out, according to Davis (1964), to identify and separate the superoxide dismutase isozymes. Measurement of SOD activity was assayed by observing the inhibition of photochemical reduction of nitro blue tetrazolium (NBT), according to the protocol of Azevedo et al., (1998). The SOD activity was observed as an achromatic band on a purple-blue background (usually within 5-7 min). For SOD isoform identification, assays were performed in the presence of selective inhibitors. KCN (3 mM) inhibits only Cu/Zn SOD; H<sub>2</sub>O<sub>2</sub> (5 mM) inhibits both Cu/Zn-SOD and Fe-SOD; Mn-SOD was not inhibited by KCN or H<sub>2</sub>O<sub>2</sub> (Azevedo et al. 1998).

**Catalase Activity:** Catalase activity was assayed by the method of Barka (2001). The reaction mixture (3 ml) consisted of enzyme extract (30  $\mu$ L), 10 mM H<sub>2</sub>O<sub>2</sub> and 50 mM potassium phosphate buffer, pH 7.0. The catalase activity was expressed as  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup> of protein.

**Ascorbate Peroxidase Activity:** APX activity was determined from the decrease in absorbance (ABS) at 290nm, due to the H<sub>2</sub>O<sub>2</sub> – dependent oxidation of ascorbate. The 2 mL reaction mixture contained 50 mM potassium phosphate (pH 7.5), 0.5 mM ascorbic acid, 1 mM H<sub>2</sub>O<sub>2</sub>, 0.1 mM EDTA and enzyme extract (30  $\mu$ L) at 25° C (Barka, 2001). The activity was expressed as nmol min<sup>-1</sup> mg<sup>-1</sup> of protein. Following electrophoretic separation (8% NATIVE-PAGE, according to Davis 1964), the gels were equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM ascorbic acid for a total of 30 min. The gels were then incubated with 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbic acid and 2 mM H<sub>2</sub>O<sub>2</sub> for 20 min. H<sub>2</sub>O<sub>2</sub> was added to this solution immediately prior to incubation with the gel. The gels were subsequently washed with 50 mM sodium phosphate buffer (pH 7.0) for 1 min and submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8), 28 mM TEMED, and 2.45 mM NBT with gentle agitation. The APX activity was observed as an achromatic band on a purple-blue background (usually

within 3-5 min). The reaction was stopped with a brief wash with deionized water (Mittler and Zilinskas, 1993).

**Guaiacol Peroxidase activity:** Peroxidase activity (Guaiacol peroxidase) with guaiacol as substrate was assayed by following the increase in absorbance at 470 nm using a modified procedure of Cakmak and Horst (1991). The assay mixture contained 25 mM phosphate buffer (pH 6.0), 2.58 mM guaiacol and 10 mM H<sub>2</sub>O<sub>2</sub> in a total volume of 1 mL. The reaction was initiated by the addition of 10  $\mu$ g of total protein and the increase in absorbance at 470 nm was measured for 5 min. The peroxidase activity was expressed as  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup> of protein. For gel activity, protein extracts were resolved by native-continuous-PAGE, pH 8.8, using 25 mM Tris, 192 glycine, pH 8.3 as running buffer. After electrophoresis, the gel was washed three times for 5 min with 25 mM phosphate buffer (pH 6.0) and incubated with the assay mixture described above. The brown colored bands (due to guaiacol polymerization) were identified after 1 min.

**In Vivo detection of H<sub>2</sub>O<sub>2</sub> in *Ricinus communis* leaves:** Diaminobenzidine (DAB) was used for the detection of H<sub>2</sub>O<sub>2</sub> staining in leaf tissues (Thordal-Christensen *et al.* 1997). After treatment with JAME, the leaves were excised and floated in 0.1% (v/v) TritonX-100 containing 1 mg ml<sup>-1</sup> DAB, and vacuum infiltrated for 30 min. DAB incubation was continued for 24 h. At the end of the staining, leaves were washed in distilled water, boiled in 95% ethanol for 10 min and examined for staining. H<sub>2</sub>O<sub>2</sub> was visualized as a reddish brown coloration.

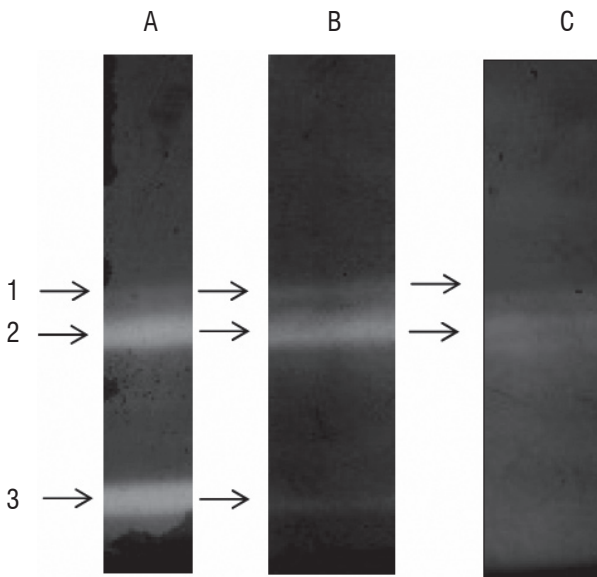
**ROS detection:** The presence of ROS was determined by DPPH radical-scavenging activity as proposed by Brand-Williams *et al.* (1995). Fresh leaves from treated or control plants were harvested into liquid nitrogen in a mortar in the presence of absolute ethanol (1:5 w/v). The homogenate was centrifuged at 15 000 *g* for 10 min at 4° C, and the supernatant obtained was used for analysis. One-hundred  $\mu$ L of the supernatant were mixed with 1mL of 6.5 x 10<sup>-5</sup>M solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol. The mixture was shaken vigorously and maintained for 30 min in the dark. The absorbance was measured at 515 nm. The absorbance of the control was obtained by replacing the sample with methanol. Rutin was used as a control. The scavenging activity was calculated using the following equation:

Scavenging activity (%) = [(A 515 of control – A515 of sample) / A 515 of control ] x 100

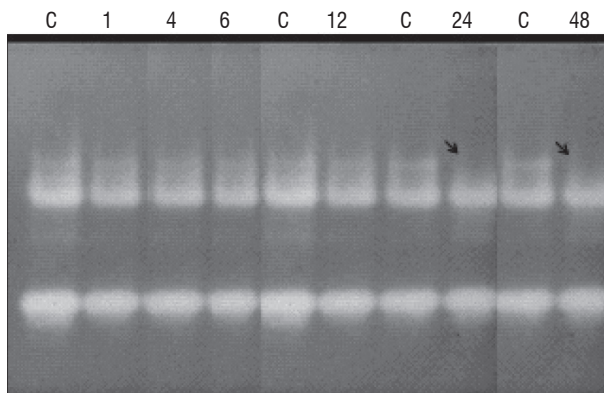
## RESULTS

In order to investigate the effect of JAME on *Ricinus communis*, plants at the three-leaf-stage (15 days) were exposed to JAME vapors and the superoxide dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase activities were evaluated in the leaf extracts.

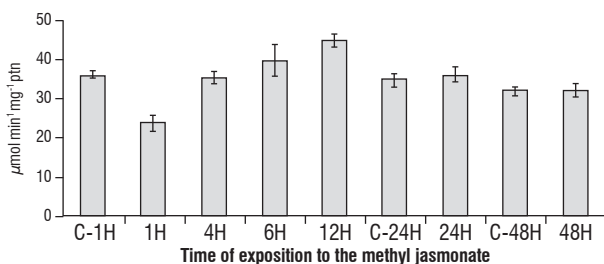
Three isoforms of SOD were detected by zymography after fractionation of crude leaf extracts by 8% NATIVE-PAGE (bands 1 - 3, Figure 1) and were identified using selective inhibitor assays. The band with the lowest mobility was not inhibited by KCN or H<sub>2</sub>O<sub>2</sub>, which classified as a Mn-SOD (band 1). H<sub>2</sub>O<sub>2</sub> inhibited the activity of the second band, which is probably a Fe-SOD and the inhibitors KCN and H<sub>2</sub>O<sub>2</sub> reduced the activity of band 3, which was identified as Cu/Zn- SOD. The treatment with JAME (0.025%) significantly decreased the activity of one Mn-SOD (band 1) after 24 and 48 h of treatment (Figure 2). Figure 3 shows that the exposure to JAME vapors decreased the catalase activity in leaf extracts, when compared with untreated control plants. The effect was stronger during the first hour of treatment and the activity returned to control levels after 12 hours. After 24 h and 48 h of treatment, no significant changes for this enzyme were observed.



**Figure 1.** Isoforms of superoxide dismutase activity determined in 8% gel electrophoresis under non-denaturing conditions. A – pre-incubated gel in buffer without inhibitor; B – gel incubated in buffer + KCN (isoform Cu/Zn-SOD inhibitor); C – gel incubated in buffer + H<sub>2</sub>O<sub>2</sub> (isoforms Cu/Zn-SOD and Fe-SOD inhibitor).

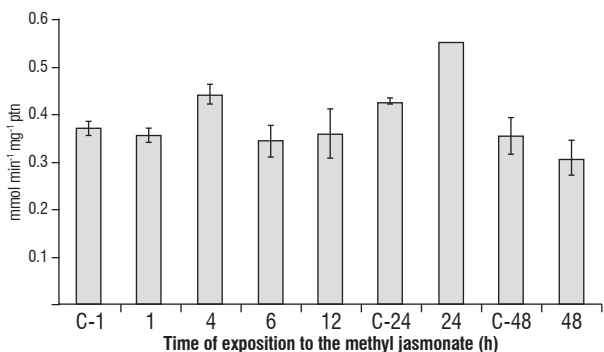


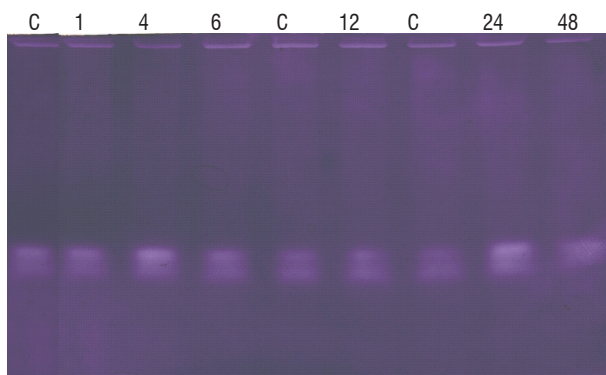
**Figure 2.** Superoxide dismutase activity determined by 8% gel electrophoresis under non-denaturing conditions. Lines: C - control; 1, 4, 6, 12, 24 and 48 – hours of exposure to methyl jasmonate. Arrows: SOD isoform altered after 24 h and 48 h of JAME treatment.



**Figure 3.** Catalase activity, determined as the decrease in absorbance at 240nm for 1 min following the decomposition of hydrogen peroxide. The reaction mixture contained 5 mM phosphate buffer (pH 7.0) and 15 mM H<sub>2</sub>O<sub>2</sub>. Data are means ± S.D. (n=3).

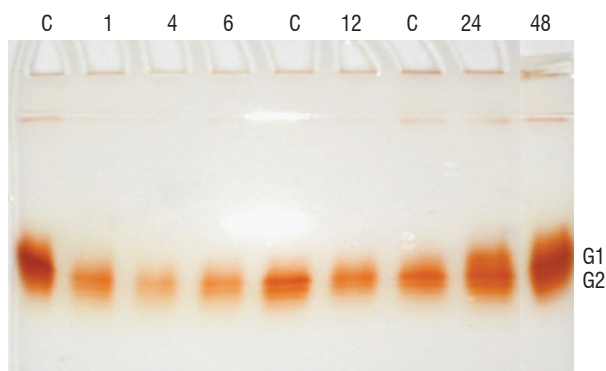
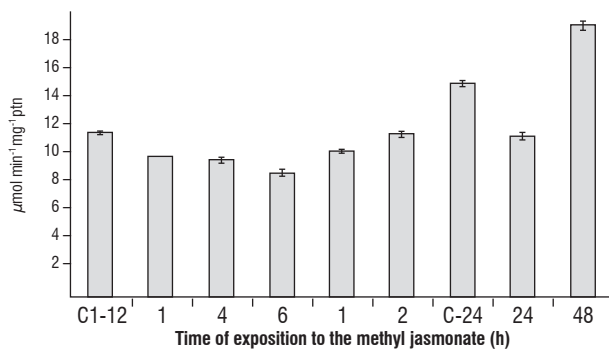
The APX activity was detected in both crude extract and directly on the gels, after fractionation of this extract by native PAGE. Figure 4A shows that the total APX activity increased after 4h and 24h of JAME treatment. A similar behavior was observed for the two isoform bands detected in the gel assay (Figure 4B).





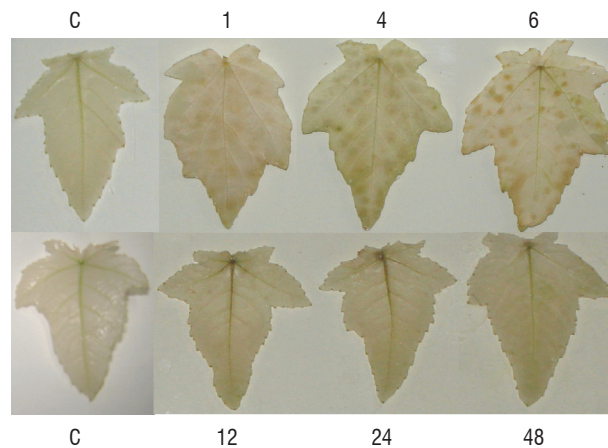
**Figure 4.** Ascorbate peroxidase activity in crude extract of plant control and treated for different times. **A)** Activity of ascorbate peroxidase, demonstrated as the decrease in the absorbance at 290 nm following ascorbate oxidation in the slurry of control and treated plants. The activity was determined as (mmol min<sup>-1</sup> mg<sup>-1</sup> of protein). Lines: C – control; 1 – 48 h: time of exposure to methyl jasmonate. Data are means ± S.D. (n=3). **B)** Activity of ascorbate peroxidase, determined in gel after separation of proteins by 8% native electrophoresis. Lines: C - control; 01-48h - time of exposure to methyl jasmonate. Black lines: APX isoforms detected.

The guaiacol activity of the crude extract decreased after 1, 4 and 6 h, and returned to near control levels after 12 h, before increasing considerably after 24 and 48 hours of treatment (Figure 5A). Two guaiacol peroxidase activities (named G1 and G2) were observed when the guaiacol activity was assayed in gel after native PAGE and both of them presented a similar behavior for the total activity observed in the crude extract (Figure 5B).



**Figure 5.** Guaiacol peroxidase activity in crude extract of control plants and plants treated for different times. **A)** Demonstrative graph of the guaiacol peroxidase activity. The activity was defined as (▲) ABS μg 470nm /ug protein x min). Data are means ± S.D. (n=3). **B)** Activity of guaiacol peroxidase, determined in gel after separation of proteins by 12% native electrophoresis. Lines: C - control; 1-48h – hours of exposure to methyl jasmonate. G1 and G2 - bands detected in gel.

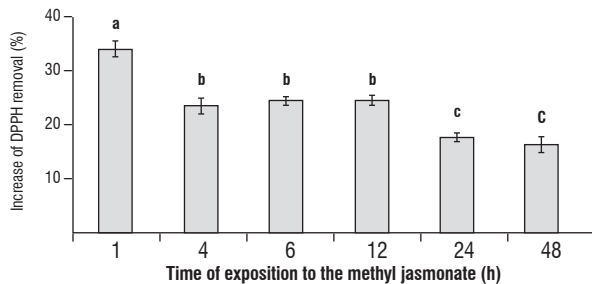
In order to investigate the oxidant status and generation of ROS in leaf tissues, we assayed the production of H<sub>2</sub>O<sub>2</sub> in *Ricinus communis* plants after exposure of JAME vapors for 1 - 48 Hours. DAB polymerizes and turns deep brown in the presence of H<sub>2</sub>O<sub>2</sub>. The intensity of the coloration and its localization can be qualitatively assessed and photographed. The development of the DAB-H<sub>2</sub>O<sub>2</sub> reaction product in *Ricinus communis* leaves, in response to JAME treatment is shown in Figure 6. The production of H<sub>2</sub>O<sub>2</sub> initiated after 1 h of treatment and intensified until 6h. These results also indicate that the H<sub>2</sub>O<sub>2</sub> production was concentrated in the major veins of the leaves after 12 and 24 h after of treatment, and that this production disappeared after 48 hours of JAME treatment.



**Figure 6.** H<sub>2</sub>O<sub>2</sub> detection in *Ricinus communis* leaves. **A)** control, 1h, 4h, 6h, control, 12h, 24h and 48h after JAME treatment, respectively. **B)** control, 1h, 4h, 6h, control, 12h, 24h and 48h after JAME treatment, respectively.

The antioxidant activity of the fractions was determined by measuring their ability to remove free radicals present in a methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Figure 7 shows the results from the antioxidant test, expressed as the percentage of DPPH free radicals removed after 30 min. All the extracts exhibited antioxidant activity. An increase of 33 % was detected in free radical removal at 1 h after JAME treatment, and 25 % was observed after 4 h, 6 h and 12 h and 13 % after 24 h and 48 h of treatment.

Positive control, rutin, removed 100% of the initial DPPH free radicals.



**Figure 7.** Detection of reactive oxygen species in the slurry of *Ricinus communis* leaves after treatment with methyl jasmonate, determined as the fold increase from control at 1 to 48h after treatment. Data are means  $\pm$  S.D. ( $n=3$ ).

## DISCUSSION

The role of JA in plants has received considerable attention and various modes of JA and JAME action have been discussed (Farmer and Ryan, 1990; Farmer, 2007). This study aimed at evaluating the ability of JAME, exogenously applied to *Ricinus communis* leaves, to counteract stress-induced responses involving antioxidative enzymes and ROS.

Compounds that are able to reduce the damaging effects of certain stresses may be of great importance from both theoretical and practical points of view. ROS are dangerous cytotoxic molecules, but also act as intermediate signaling molecules to regulate the expression of genes associated with antioxidant defense mechanisms (Vranova et al., 2002; Neill et al., 2002). To counteract the toxicity of reactive oxygen species, plants have developed a highly efficient antioxidant enzymes defense system, mainly including SOD, APX, CAT and Prx, increasing tolerance to different stress factors (Jiang and Zhang 2002).

In our studies, three SOD isoforms were found (Mn-SOD, Fe-SOD and Cu/Zn-SOD), however just one presented alteration in its activity after JAME treatment. JAME resulted in a significant decrease in the activity of one Mn-SOD isoform, compared to the control, in *R. communis* leaves, 24 and 48 h after treatment. Similar results were observed by Lijun et al. (2005), who reported inhibition of SOD by high concentrations of cadmium. In addition, Hernández et al. (1993) demonstrated that saline stress causes a reduction of Mn-SOD and Cu/Zn-

SOD in susceptible cultivars of *Pisum sativum*. In *Arabidopsis thaliana*, the total SOD activity decreased three days after JAME treatment and increased two fold after seven days of treatment (Jung, 2004).

Furthermore, in the present study, catalase activity showed a transient behavior at 1 h to 12 h of JAME treatment and then remained at a level similar to that of the control. The catalase reduction at 1 h after treatment could be related to an accumulation of  $H_2O_2$  in the peroxisomes, as a strategy to preserve it from total degradation. Thus,  $H_2O_2$  could be used as a defense signaling molecule in other parts of the plant (Thordal-Christensen et al., 1997). Therefore, an increase in CAT activity 12 h after JAME treatment and its return to control levels at 24 and 48 h after treatment could be related to a negative feedback, where the JA and JAME could be stabilized. Other reports have registered the decrease in catalase activity, as in nodules of *Phaseolus vulgaris* roots under saline stress, whereas in other parts of the roots, CAT activity was not detected (Jebara et al., 2005). Chamnongpol et al. (1998) reported catalase as a mediator of defense response, because the reduction in its activity was related to the accumulation of  $H_2O_2$  in tobacco. Jung (2004) verified that catalase activity did not modify between the 3<sup>rd</sup> and 5<sup>th</sup> day after exposure to treatment with methyl jasmonate, but increased by 58%, seven days after the treatment in *A. thaliana*. Depending on the concentration of jasmonic acid used in the induction of the defense response, the activity of antioxidant enzymes is modified in a distinct manner. In *Morinda elliptica*, catalase activity was observed to increase after 1 day and was then drastically reduced after 3 and 6 days of treatment with 50  $\mu$ M JA.

When JA was used at a concentration of 100  $\mu$ M, no significant alteration in the enzyme was observed until the third day, but the activity was reduced after 6 days of treatment (Chong et al. 2005). The catalase activity reduction was also registered in *Catharanthus roseus* cultures, induced by fungi (Zhao et al. 2001) and by water stress in strawberry (Wang, 1999).

We also observed that APX activity increased 4 and 24h after treatment with JAME in *Ricinus communis* leaves. However, the enzyme does not suffer alterations, maintaining its activity at a level similar to that of controls in a period of 1, 6, 12 and 48 h subsequent to treatment. Chong et al., (2005) also verified a transient behavior of APX in *Morinda elliptica*,

in which the ascorbate peroxidase activity did not suffer any alteration 1 day after 50  $\mu$ M JAME treatment, however, it increased at 3 days and reduced drastically at 6 days after treatment with JAME. These authors also observed an increase in APX activity after 3 days of 100  $\mu$ M JAME treatment and a reduction of 50% in the activity after 6 days of treatment.

Guaiacol is often used as a substrate for the measurement of peroxidase activity (Hiraga et al., 2001). The enzyme, guaiacol peroxidase, is involved in protective actions in plants, participates in the lignification of the cellular wall, biosynthesis of ethylene, reestablishment after wound and defense against pests (Gazaryan et al. 1996). In our study, GPX presented a gradual decrease in activity until 6 h and a reestablishment of control values 12 h after the treatment. After this period, the activity increases significantly, reaching nearly 50% at 48 h after JAME treatment. The gel analysis of GPX revealed an activity behavior similar to that obtained by crude extract analysis. Jung (2004) reported that GPX activity increases by about 100 fold after seven days of treatment with JAME in *A. thaliana* and suggested that most of the protection against oxidative stresses in plants treated with JAME may be the result of enzymatic antioxidant substances, particularly GPX. Our results indicate a gradual and significant increase in GPX activity at 24 and 48 h, however the reduced activity of this enzyme at the initial moments after the treatment with JAME may be due to the generation of ROS, possibly involved with the mediation of stress defense signaling. The delayed increase in GPX activity is probably related to the return to normal metabolism. Jebara et al. (2005) showed the activity reduction of three GPX isoforms (one acid isoform and two basic ones) in roots of *Phaseolus vulgaris*, while in the nodules, a reduction of activity GPX occurs under saline stress, suggesting that GPX has an important role in detoxify H<sub>2</sub>O<sub>2</sub> under saline stress.

The variation in antioxidant activity within the cell can modify the ROS content and lead to death or an acclimatization response. With the objective of verifying whether the alteration in the antioxidant status in *Ricinus communis* is in accordance with variations in the ROS formation, we carried out experiments to detect the H<sub>2</sub>O<sub>2</sub> content and free radicals. A gradual accumulation of H<sub>2</sub>O<sub>2</sub> was observed to occur between 1 and 6h after the treatment with JAME. In this period, the staining with DAB disclosed circular injuries that spread over the surface of the leaf. At 12 and 24h after the treatment, DAB

staining was less intense and was restricted to the central vein and, at 48h, there was no apparent formation of H<sub>2</sub>O<sub>2</sub>.

Other studies have documented the hydrogen peroxide production in stress. Rao et al. (1997) observed that salicylic acid (SA) increases the production of H<sub>2</sub>O<sub>2</sub>, the lipidic peroxidation and the oxidative damage to the proteins in *A. thaliana*. Cho and Seo (2005) related an increase of H<sub>2</sub>O<sub>2</sub> content in *A. thaliana* treated with Cadmium. Our data referring to the detection of free radical content, in leaves treated with methyl jasmonate, complement results regarding the production of H<sub>2</sub>O<sub>2</sub> in *R. communis* leaves. An increase of 33% was observed to occur in the content of free radicals at 1h after treatment with JAME; this value decreased, returning to control levels by 48h. Apparently, the sharp formation of ROS at the initial moments of JAME exposure is related to the decrease in the activities of CAT and GPX.

In conclusion, the modification of antioxidants enzymes (SOD, APX, GPX, CAT) can play important protective roles avoiding the deleterious effects triggered by elevated levels of ROS observed at initial moments after JAME exposure. In general, plant defense signaling is studied using model plants. However, different plant species probably have particularities in their responses. Therefore, reinforce the relevance to study plant defense signaling in *Ricinus communis*, an important tropical oil-plant and a promising bioenergy source.

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## REFERENCES

- Azevedo RA, Alas RM, Smith RJ, Lea PJ (1998) Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. *Physiol. Plant.* 104:280–292.
- Barka EA (2001) Protective enzymes against reactive oxygen species during ripening of tomato (*Lycopersicon esculentum*) fruits in response to low amounts of UV-C. *Aust. J. Plant Physiol.* 28:785–791.
- Becana M, Paris FJ, Sandalio LM, del Rio LA (1989) Isoenzymes of superoxide dismutase in nodules of *Phaseolus vulgaris* L.; *Pisum sativum* L.; and *Vigna unguiculata* (L.) Walp. *Plant Physiol.* 90:1286–92.
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT Food Sci. Technol.* 28:25–30.
- Cakmak I, Horst WJ (1991) Effect of aluminum on lipid-peroxidation, superoxide-dismutase, catalase, and peroxidase-activities in root-tips of soybean (glycine-max). *Physiol. Plant.* 83:463–468.

- Chamnonngpol S, Willekens H, Moeder W, Langebartels C, Sandermann H Jr, Van Montagu M, Inze D, Van Camp W (1998) Defense activation and enhanced pathogen tolerance induced by H<sub>2</sub>O<sub>2</sub> in transgenic tobacco. *Proc. Natl. Acad. Sci. USA.* 95:5818–5823.
- Cho UH, Seo NH (2005) Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. *Plant Sci.* 168:113–120.
- Chong TM, Abdullah MA, Fadzillah NM, Lai OM, Lajis NH (2005) Jasmonic acid elicitation of anthraquinones with some associated enzymic and non enzymic antioxidant responses in *Morinda elliptica*. *Biochem. J.* 36:469–477.
- Dalton DA, Joyner SL, Becana M, Iturbe-Ormaetxe I, Chatfield JM (1998) Antioxidant defences in the peripheral cell layers of legume root nodules. *Plant Physiol.* 116:37–43.
- Davis BJ (1964) Disc electrophoresis, part II. *Ann. NY. Acad. Sci.* 121:404–427.
- Farmer EE (2007) Plant biology - Jasmonate perception machines. *Nature.* 448:659–660.
- Farmer EE, Ryan CA (1990) Interplant communication - airborne methyl jasmonate induces synthesis of proteinase-inhibitors in plant-leaves. *Proc. Natl. Acad. Sci. USA.* 87:7713–7716.
- Faurie B, Cluzet S, Merillon, JM (2009) Implication of signaling pathways involving calcium, phosphorylation and active oxygen species in methyl jasmonate-induced defense responses in grapevine cell cultures. *J. Plant Physiol.* 166:1863–1877.
- Gazaryan IG, Lagrimini LM, Ashby GA, Thorneley RNF (1996) Mechanism of indole-3-acetic acid oxidation by plant peroxidases: anaerobic stopped-flow spectrophotometric studies on horseradish and tobacco peroxidases. *Biochem. J.* 313:841–847.
- Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. *Plant Cell Physiol.* 42:462–468.
- Hernández JA, Corpas FJ, Gómez M, Rio LA, Sevilla F (1993) Salt-induced oxidative stress mediated by activated oxygen species in pea leaf mitochondria. *Physiol. Plant.* 89:103–110.
- Hossain Z, Lopez-Climent MF, Arbona V, Perez-Clemente RM, Gomez-Cadenas A (2009) Modulation of the antioxidant system in citrus under waterlogging and subsequent drainage. *J. Plant Physiol.* 166: 1391–1404.
- Jebara S, Jebara M, Limam F, Aouani ME (2005) Changes in ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase activities in common bean (*Phaseolus vulgaris*) nodules under salt stress. *J. Plant Physiol.* 162:929–936.
- Jiang MY, Zhang JH (2002) Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. *J. Exp. Bot.* 379:2401–2410.
- Jubany-Mari T, Prinsen E, Munne-Bosch S, Alegre L (2010) The timing of methyl jasmonate, hydrogen peroxide and ascorbate accumulation during water deficit and subsequent recovery in the Mediterranean shrub *Cistus albidus* L. *Environ. Exp. Bot.* 69:47–55.
- Jung S (2004) Effect of chlorophyll reduction in *Arabidopsis thaliana* by methyl jasmonate or norflurazon on antioxidant systems. *Plant Physiol. Biochem.* 42:225–231.
- Kumari GJ, Reddy AM, Naik ST, Kumar SG, Prasanthi J, Sriranganayakulu G, Reddy PC, Sudhakar C (2006) Jasmonic acid induced changes in protein pattern, antioxidative enzyme activities and peroxidase isozymes in peanut seedlings. *Biol. Plant.* 50:219–226.
- Lijun L, Xuemei L, Yaping G, Enbo M (2005) Activity of the enzymes of the antioxidative system in cadmium-treated *Oxya chinensis* (*Orthoptera Acridoidea*). *Environ. Toxicol. Pharmacol.* 20:412–416.
- Ma ZL, Gao KS (2010) Spiral breakage and photoinhibition of *Arthrospira platensis* (Cyanophyta) caused by accumulation of reactive oxygen species under solar radiation. *Environ. Exp. Bot.* 68:208–213.
- Matamoros MA, Dalton DA, Ramos J, Clemente MR, Rubio MC, Becana M (2003) Biochemistry and molecular biology of antioxidants in the rhizobia-legume symbiosis. *Plant Physiol.* 133:499–509.
- Miller G, Shulaev V, Mittler R (2008) Reactive oxygen signaling and abiotic stress. *Physiol. Plant.* 133:481–489.
- Mittler R, Zilinskas BA (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. *Anal. Biochem.* 212:540–546.
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7:405–410.
- Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.* 9:490–498.
- Neill SJ, Desikan R, Clarke A, Hurst RD, Hecock JT (2002) Hydrogen Peroxide and nitric oxide as signaling molecules in plants. *J. Exp. Bot.* 53:1237–1247.
- Noctor G, Foyer CH (1998) Ascorbate and glutathione keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:249–79.
- Parra-Lobato MC, Fernandez-Garcia N, Olmos E, Alvarez-Tinaut MC, Gomez-Jimenez MC (2009) Methyl jasmonate-induced antioxidant defence in root apoplast from sunflower seedlings. *Environ. Exp. Bot.* 66: 9–17.
- Pearce G, Moura D, Stratmann J, Ryan CA (2001) Production of multiple plant hormones from a single polyprotein precursor. *Nature*, 411: 817–820.
- Rao MV, Paliyath G, Ormrod DP, Murr DP, Watkins, CB (1997) Influence of salicylic acid on H<sub>2</sub>O<sub>2</sub> production, oxidative stress, and H<sub>2</sub>O<sub>2</sub> -metabolizing enzymes. *Plant Physiol.* 115:137–149.
- Tanou G, Molassiotis A, Diamantidis, G (2009) Induction of reactive oxygen species and necrotic death-like destruction in strawberry leaves by salinity. *Environ. Exp. Bot.* 65:270–281.
- Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H<sub>2</sub>O<sub>2</sub> in plants. H<sub>2</sub>O<sub>2</sub> accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* 11:1187–1194.
- Vranova E, Inze D, Van Breusegem F (2002) Signal transduction during oxidative stress. *J. Exp. Bot.* 53:1227–1236.
- Wang SY (1999) Methyl jasmonate reduces water stress in strawberry. *Plant Growth Regul.* 18:127–134.
- Zhao J, Zhu W, Hu Q (2001) Enhanced catharanthine production in *catharanthus roseus* cell cultures by combined elicitor treatment in shake flasks and bioreactors. *Enzyme Microb. Technol.* 28:673–681.