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Soluble and cell wall bound peroxidase activities are markers of flower bud development stages in lemon (*Citrus limon* L.)

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Summary

Specific activities of H_2O_2 scavenging enzymes, soluble peroxidase (G-POD), catalase (CAT) and ascorbate peroxidase (APX), and cell wall bound (CWB) G-POD, have been characterized in flower buds and leaves in potted lemon trees (*Citrus limon* L. Burm.) growing in a glasshouse in Tunisia. In leaves during the winter rest period (from September to December), enzyme activities did not change markedly. Then, leaf G-POD (soluble and CWB activity) increased 4-5 fold in January as the buds reached dormancy release. In growing buds, the activities of the three enzymes increased strongly (10- to 100-fold) between dormancy and full bloom and decreased slightly (3-fold) post-bloom (beginning of fruit-set). Along the shoots, the developmental stages of flower buds advanced from node 1 to node 5. G-POD activity increased from the shoot apex toward the base of the twig in both leaves and buds. A similar trend was observed for APX and CAT but their activities tended to decrease slightly farther from the apex. Retardation of flowering by application of growth regulators strongly reduced leaf-specific activities of the H_2O_2 scavenging enzymes. Therefore, G-POD activity (and to a lesser extent APX and CAT) appears to be a good marker of flower formation in lemon (from bud swelling to full bloom). The results also suggest a link between H_2O_2 production and flower bud development.

Abbreviations
CWB: Cell wall bound; ROS: Reactive oxygen species; G-POD: Guaiacol peroxidase; APX: Ascorbate peroxidase; CAT: Catalase; GA_3: Gibberellic acid; MH: Maleic hydrazide.

Introduction

Flowering is an essential phenomenon in the life cycle of higher plants, being the central process of species reproduction. The formation of flower buds is also a fundamental factor of productivity. The change from vegetative to reproductive growth is called the flowering transition. It is controlled by inheritance, internal and external factors and is accompanied by various changes in the biochemistry, physiology, cytology and morphology of the buds leading to the formation of reproductive structures (Chen et al., 2009). The lemon (*Citrus limon* L.) is an economically important crop consumed worldwide. The flowering process in citrus consists of several discrete phases: flower bud induction, bud differentiation and anthesis. The induction process is dependent on environmental and endogenous (such as fruit load) factors, and results in the commitment of meristematic cells to form reproductive structures. Floral differentiation involves the anatomical and morphological transition of vegetative meristems to floral meristems (Davenport, 1990). In citrus, the differentiation of floral organs begins during the first stages of bud swelling and sprouting. Lemon trees have two major flowering periods in Mediterranean climates. One is in spring (following the inductive cold winter season (Garcia-Luis and Kanduser, 1995) and plays the major role in yield. The second corresponds to the autumn-flush growth. However, the lemon tends to flower continuously throughout the year in cooler, coastal climates where trees can produce several crops each year. Flowering shoots are often produced on woody twigs of the previous year's spring-flush of growth but may also be produced on summer-flush twigs or on older wood (Spiegel-Roy and Goldschmidt, 1996). They may form only flowers (generative shoots also called leafless inflorescences) or both flowers and leaves (mixed-type inflorescences).

In aerobic life, oxygen is subject to *in vivo* activation into toxic forms during normal cell metabolism. Reactive oxygen species (ROS) are partially reduced oxygen intermediates. The most damaging forms of ROS are superoxide radicals ($*O^-_2$), hydroxyl radicals (*OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) and ROS are generated during metabolic conversion of molecular oxygen (Cerutti, 1991). In plants, the production of ROS is drastically increased in response to biotic and abiotic stresses. The ROS may be very damaging since they can oxidize a variety of organic molecules such as proteins, lipids and DNA (Pietta, 2000) so their removal is essential. Plants possess well-defined antioxidative systems to scavenge ROS, including low-molecular weight compounds and enzymes (Hossain et al., 2006). Superoxide dismutase, SOD (EC 1.15.1.1) detoxifies $*O^-_2$ by converting it into O_2 and H_2O_2. While H_2O_2 is not itself a free-radical, it can generate highly reactive *OH radicals. Catalase, CAT (EC 1.11.1.6) catalyses H_2O_2 breakdown into H_2O and O_2. Peroxidases, POD (EC 1.11.1.7), catalyse oxido-reduction between H_2O_2 and various reductants with low substrate specificity; ascorbate peroxidase, APX (EC 1.11.1.11) also degrades H_2O_2 requiring ascorbic acid as electron donor.

APX, CAT and POD are among the most important enzymes in H_2O_2 scavenging. APX acts as a central component of the major hydrogen peroxide detoxifying system in plant cells: the ascorbate – glutathione cycle, and plays an essential role in the control of intracellular ROS levels (Rosa et al., 2010). CAT participates in the main defense system against the accumulation and toxicity of hydrogen peroxide and plays a central role in controlling H_2O_2 levels in the cell, therefore controlling the regulation of the signal transduction pathway that is dependent on ROS (Starzynska et al. 2003). It has been proposed that the levels of POD are strongly modulated in cells since they are involved in growth, development and senescence processes in plants and vary in response to both biotic and abiotic stresses (Noriega et al., 2007). In recent years, there has been growing interest in the functional significance of ROS in plant growth and development. ROS are known not only for their hazardous oxidative damage effects but also for their role in modulating both signaling and defense functions. It has been shown that H_2O_2 acts as a development signaling molecule (Tian et al., 2003) and that H_2O_2 metabolism coordination in different sub-cellular compartments is complex since it can diffuse between compartments (Rosa et al., 2010).

Gonzalez et al. (1991) and Legrand and Bouazza (1991) showed that specific peroxidase isoenzymes were related to organogenesis.

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WANG et al. (1991, (a) and (b)) examined 'York Imperial' apple vegetative bud development prior to flowering. They observed an increase in catalase activities during bud swelling and then a decline at bud break. These authors also found high POD activity in dormant buds, which then declined during bud break to increase again during bud growth. The works of BERNAL et al. (1993) reveal that *Capsicum annuum* flowering is regulated by internal factors associated with an increase in peroxidase activity in the leaves. ABASSI et al. (1998), evaluating Red Spur Delicious flower bud development, report that changes in catalase and peroxidase activities were related to growth and development of the apple flower. Also, MONERRI and GUARDIOLA (2001) showed that changes in peroxidase activity occur concomitantly with floral transition in *Citrus unshiu*. HUFF and DYBING (1980), in the soybean (*Glycine max*) observed that peroxidase activity changes in parallel with ovary development, increasing rapidly in growing pods but not in shedding flowers. Peroxidases could be involved in numerous processes associated with flower bud development: lignification (WHETTEN et al., 1998), suberization (ESPELIE et al., 1986), cross-linking of cell wall structural proteins (FRY, 1986), auxin catabolism (LAGRIMINI et al., 1997) and tissue senescence (ABELES et al., 1988).

The literature thus suggests that the enzymes which degrade H_2O_2 (in particular POD) play an important role in protecting plant tissue during flower-bud development. However, reports are scarce, and often contradictory, so changes in these enzyme activities in relation to flowering must be clarified. In addition, very little (if any) information is available on enzymatic antioxidant activity in lemon during bud development although it has been reported that flowering can be associated with an increase in POD activity in the leaves (BERNAL et al., 1993). Thus, the present study aimed to evaluate changes in POD, APX and CAT (H_2O_2 scavenging enzymes) activity in developing flower buds and neighboring leaves during the main flowering season in lemon. Leaf insertion level on the twigs and floral bud development stages were taken into account. Proteins are essential constituents of plant cell walls and are involved in cell division, enlargement and differentiation. Class III peroxidases (POD, EC 1.11.1.7) are abundant extracellular proteins and changes in cell wall POD activity have been associated with growth and organogenesis (ABASSI et al., 1998). Thus, we also focused on weakly bound cell wall (CWB) POD activity in addition to soluble-protein-associated POD activity. Because flowering is controlled by internal and external factors, we used growth regulators to retard flowering and distinguish between the effects of endogenous and environmental factors on H_2O_2 scavenging enzyme activity. The study provides insight into flowering behavior by finding suitable indicators of bud development.

Materials and methods

Plant material and growth conditions

The experiment was carried out on twenty uniform sized 4-year-old lemon (*Citrus limon* L. Burm, cv 'Eureka') trees grafted on *C. aurantium* rootstock at the Higher Institute of Agronomy of Chott Meriem-Tunisia (35°55'N, 10°38'E, 15 m above sea level). The trees were grown in a glasshouse in 25-litre pots containing a sandy-clay textured soil. The plants were irrigated twice a week with water at field capacity. "Mixed-type inflorescences", i.e. shoots bearing both flower buds and leaves were used in the study.

The main study began mid September i.e. 24 weeks before full bloom (FB which occurred at the end of February) in order to cover the inductive "cold" season (GARCIA-LUIS and KANDUSER, 1995). Changes in leaf protein content and enzyme activities before floral bud break were analyzed in younger fully expanded leaves sampled on one-year-old woody twigs. Changes in protein content and enzyme activity at various stages of flower bud development and fruit-set were followed in buds sampled at the beginning of March on the first node of the tip of twigs formed the previous year. Changes in protein content and enzyme activities in leaves and young growing flower buds according to the position along the shoot were assayed in young growing buds and younger fully expanded leaves sampled at the beginning of March from the tip (first insertion level, first node) to the fifth node below the apex (insertion level 5).

In many fruit trees, flowering can be inhibited by the application of exogenous GA_3. MH is a synthetic compound which has also a plant growth regulating action. It has long been used in agriculture as a depressant of plant growth which retards flowering and prolongs dormancy. A separate experiment was conducted to test the influence of GA_3 and MH on changes in leaf enzyme activities associated with the retarding effect on flowering. Retardant sprays or water sprays (control) were applied to whole trees (5 plants per treatment) on 15th January (i.e. about 2-3 weeks before normal blooming). Treated-trees were sprayed to the drip point with GA_3 concentrations of 50 or 100 ppm and MH concentrations of 1000 or 1500 ppm (plus 0.2% Tween-80). Responses to GA_3 and MH were recorded in leaves at the beginning of February. In addition, identical treatments were applied to lemon cultivars other than "Eureka" (Femminello, Kutdeken, Santateresa and Interdonato) on 5-year-old trees cultivated outdoors in Grombalia (36°36'N, 10°30'E, 48 m above sea level).

Protein extraction

All samples were prepared for soluble and cell-wall-bound protein and enzyme analyses by grinding in liquid nitrogen. To obtain the enzyme extract, 300 mg of fresh buds or fresh leaves were homogenized in 5 ml of cold K_2HPO_4-PVP (4%) buffer (50 mM, pH 7.8). The homogenate, after filtration, was centrifuged at 12000 g for 15 min at 4 °C. The supernatant was immediately used for the assay of soluble proteins and the determination of enzyme activities. After two washings with buffer, the precipitate was extracted and centrifuged for 30 min (12 000 g at 4 °C) three times with 1M KCl in K_2HPO_4-PVP buffer (50 mM, pH 7.0). The combined supernatants were used for the assay of weakly cell wall bound protein (JAMET et al., 2006) and G-POD enzymatic activity. All preparations were performed at 4 °C. Soluble and cell wall bound proteins were determined according to BRADFORD (1976), using bovine serum albumin for calibration.

Enzyme assays

All spectrophotometric analyses were conducted in a 1.5 ml reaction mixture containing enzyme extract (10 µg of protein) with a Secomam, S100-149 UV/Visible Light spectrophotometer at ambient temperature. Guaiacol (*o*-methoxy phenol) is commonly used as substrate for the measurement of POD activity (HIRAGA et al., 2001): the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 100 mM H_2O_2, 1% guaiacol and enzyme extract. Guaiacol POD (G-POD) activity was measured by monitoring the increase in absorbance at 470 nm during polymerisation of guaiacol into tetraguaiacol (extinction coefficient 26.6 mM^{-1} cm^{-1}) according to CHANCE and MAEHLY (1955). For APX, the oxidation of ascorbic acid was monitored as the decrease in absorbance of the substrate at 290 nm (NAKANO and ASADA, 1981). The reaction mixture contained potassium phosphate buffer (50 mM, pH 7.8), sodium ascorbate (0.5 mM) and enzyme extract. The reaction was started by adding 150 µL hydrogen peroxide (100 mM) and the absorbance decrease was recorded for 1 min at 25 °C (extinction coefficient of 2.8 mM^{-1} cm^{-1}). Correction was made for the low, non-enzymatic oxidation of ascorbate by hydrogen peroxide. CAT activity was measured by following the consumption of H_2O_2 at 240 nm according to AEBI (1984), in potassium phosphate buffer (50 mM, pH 7.8) containing

100 mM H_2O_2 and enzyme extract. The reaction started when the H_2O_2 was added. All enzyme activities are expressed as specific activity (on a soluble or CWB protein basis).

Statistics

Data represent mean +/- standard error of 5 replicates (four trees were used to sample leaves and buds for each replicate). Statistical analysis was performed by ANOVA and checked for significant probability ($p \leq 0.05$) level using Sigmastat® 2.03 Statistical Software. The difference between the means was determined using Fisher's Least Significant Difference at a probability $p \leq 0.05$.

Results

Changes in leaf protein content and enzyme activities before floral bud break

During the inductive "cold" period, leaf CWB protein content (0.4 mg g^{-1} leaf FW) was on average 20-fold lower than that of the soluble fraction (8 mg g^{-1} leaf FW, Fig. 1). Low levels of protein (both soluble and CWB) were observed 22 and 20 (FB-22 and FB-20, October) and 10 (FB-10, December) weeks before FB. Specific G-POD activity was similar or slightly higher in CWB versus soluble proteins. The activity was strongly increased at the beginning of bud sprouting, 6 weeks before FB (mid January). In soluble proteins, APX activity increased by 50 per cent from September (FB-24) to October (FB-20), then it decreased reaching at FB-17 (November) a value similar to that of September. Thereafter, it rose slowly again until January. CAT activity displayed changes similar to APX except at FB-6 (release of bud dormancy) when it was lower. Overall, except in January (FB-6), changes in protein content were opposite to changes in enzyme activities.

Changes in protein content and enzyme activity at various stages of flower bud development and fruit-set

All the developmental stages studied were found on the same twig; they are shown in Fig. 2. Stage (A) shows a bud having pollen mother cells before prophase of first meiotic division. Stage (B) shows a bud during meiosis in anthers. Stage (C) shows a bud at young pollen

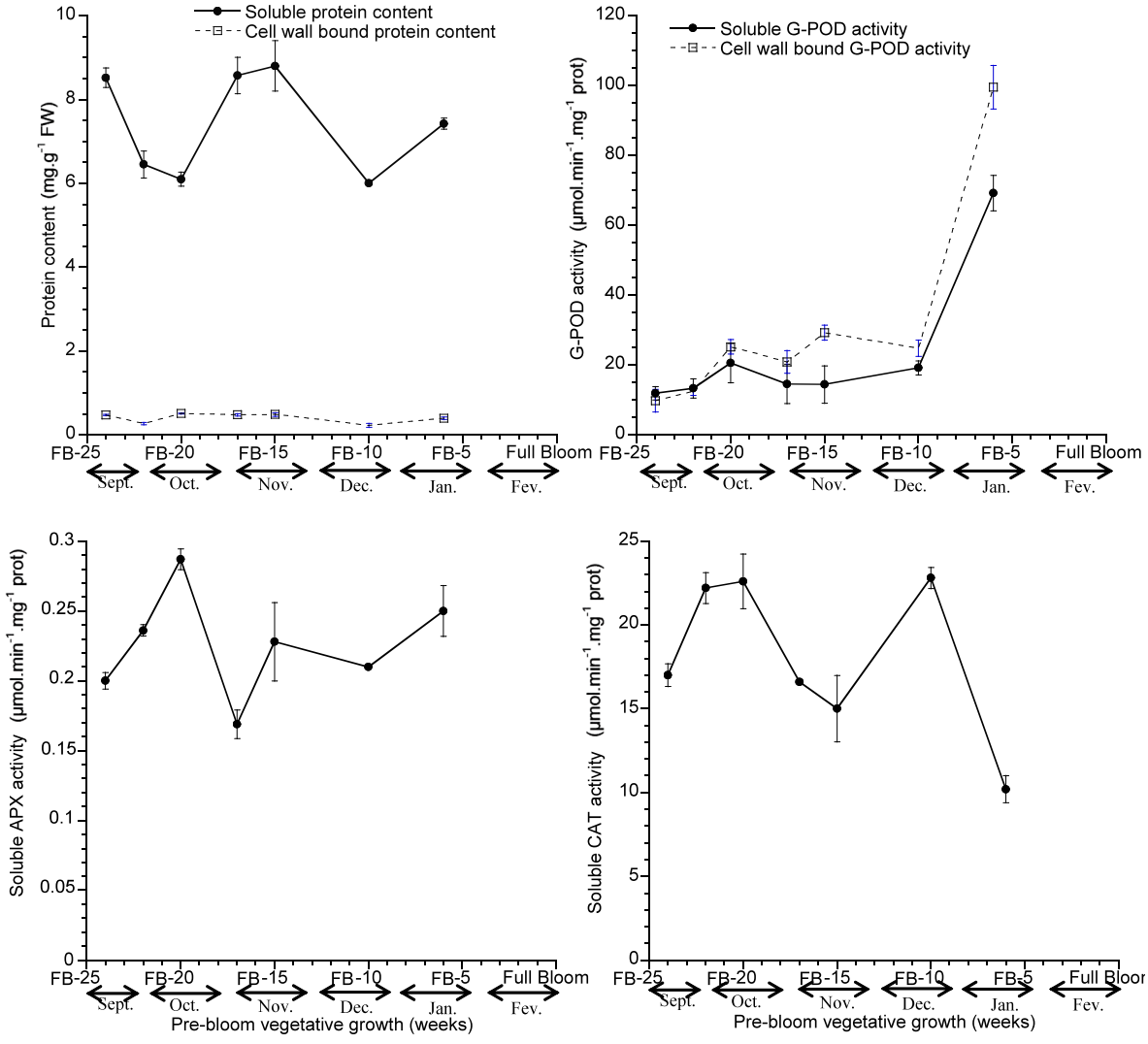

Fig. 1: Changes in biochemical parameters in younger fully expanded leaves sampled on one-year-old woody twigs bearing both leaves and future flower buds (mixed-type inflorescences) from 4-year-old lemon trees. Total soluble and cell-wall bound (CWB) proteins, and G-POD, APX and CAT specific activities and CWB G-POD specific activities were characterized during the winter resting period from September to January (release of flower bud dormancy). The sample dates reported on the x-axis are also converted into "weeks before Full Bloom (FB, end of February)". Bars indicate the standard error (mean+/- S.E., n=5).

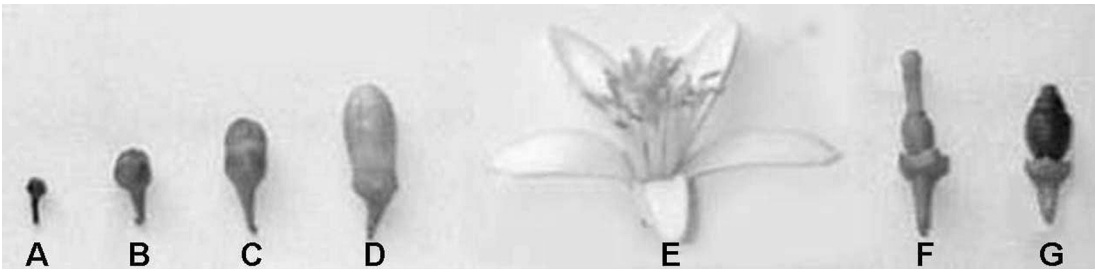

Fig. 2: Stages of flower bud development: (A) Bud having pollen mother cells before prophase of first meiotic division; (B) Bud during meiosis in anthers; (C) Bud at young pollen grain stage; (D) Bud nearly full-grown; (E) Flower at time of pollination; (F) Pistil just after fall of petals and stamens (about 6 days after pollination); (G) Ovary just after abscission of style (about 10 to 15 days after anthesis).

grain. Stage (D) shows a bud nearly full-grown. Stage (E) represents flower at time of pollination. Stage (F) shows a pistil just after fall of petals and stamens (about 6 days after pollination). And stage (G) represents the ovary just after abscission of the style (about 10 to 15 days after anthesis).

As observed in leaves, i) changes in soluble and CWB proteins in growing buds followed the same pattern and ii) soluble proteins were more abundant than CWB proteins (Fig. 3). Protein concentrations in buds beginning to swell (stage A) and in young growing buds (stages B and C) were higher than in older developmental stages and 4-5 times higher than in leaves. They reached a maximum at stage B (37 and 3 mg g^{-1} FW for soluble and CWB proteins, respectively). Then the concentration decreased to minimal at stage E (full-bloom, 5 and 0.4 mg g^{-1} FW for soluble and CWB proteins, respectively). Thereafter, it remained almost constant (fruit-set).

Specific G-POD activities were higher in CWB than in soluble proteins but they followed similar changes in both types of proteins. In soluble proteins, the three enzyme activities (G-POD, APX and CAT) displayed the same pattern of variation: the initial very low activity at bud dormancy release increased strongly during bud growth (10- to 100-fold) to peak at full bloom (stage E) and then rapidly declined at fruit-set.

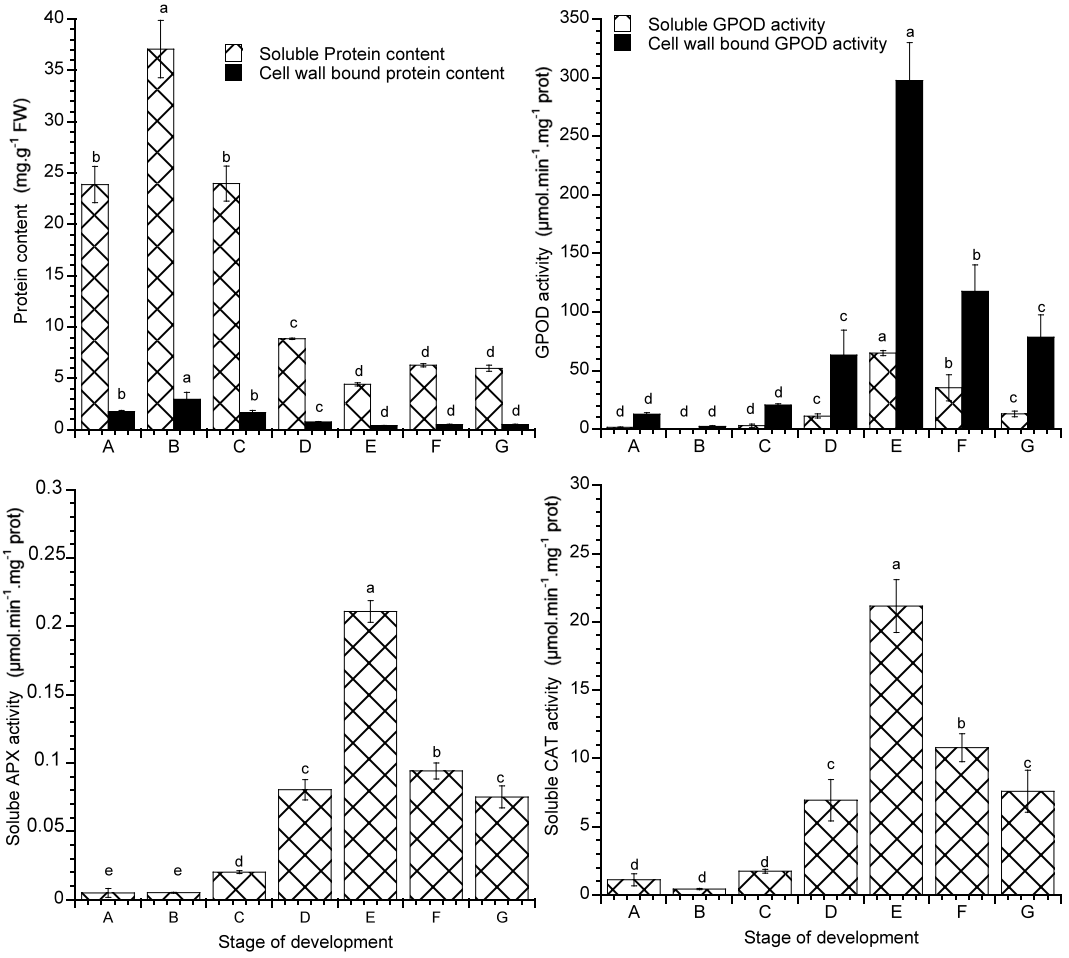

Fig. 3: Changes in protein (soluble and CWB) content and H_2O_2 scavenging enzyme specific activities in floral buds during flower development in lemon trees. Stages of development are described in Fig. 2. Bars indicate the standard error (mean+/- S.E., n=5).

Changes in protein content and enzyme activities in leaves and young growing flower buds according to the position along the shoot

Stages of development for sampled bud were: B for insertion level 1, C for insertion level 2, D for insertion levels 3 to 5. Both soluble and CWB proteins were more abundant in buds than in leaves at the first insertion level (Fig. 4). For levels 2-5, protein concentrations were similar in both organs and the values did not vary between nodes except for CWB proteins of node 5, which were less abundant than in other nodes.

Specific G-POD activities were higher in CWB than in soluble proteins in both leaves and buds. These activities were higher in buds than in leaves (between 3 and 10-fold) except in node 1 where activities were similar. In both organs, the activities increased markedly towards the base of the shoot (more than 10-fold in buds for example). In contrast to G-POD, APX and CAT activities were often greater in leaves as compared to buds. In leaves, APX activities were higher far from the shoot apex than near it (2-fold). In buds, the pattern was less clear with very low activities at the top with the highest for insertion levels 2 and 3. CAT activities showed changes similar to APX activities: in leaves CAT activities increased with the position along the shoot (2-3 fold from insertion levels 1-2 to insertion

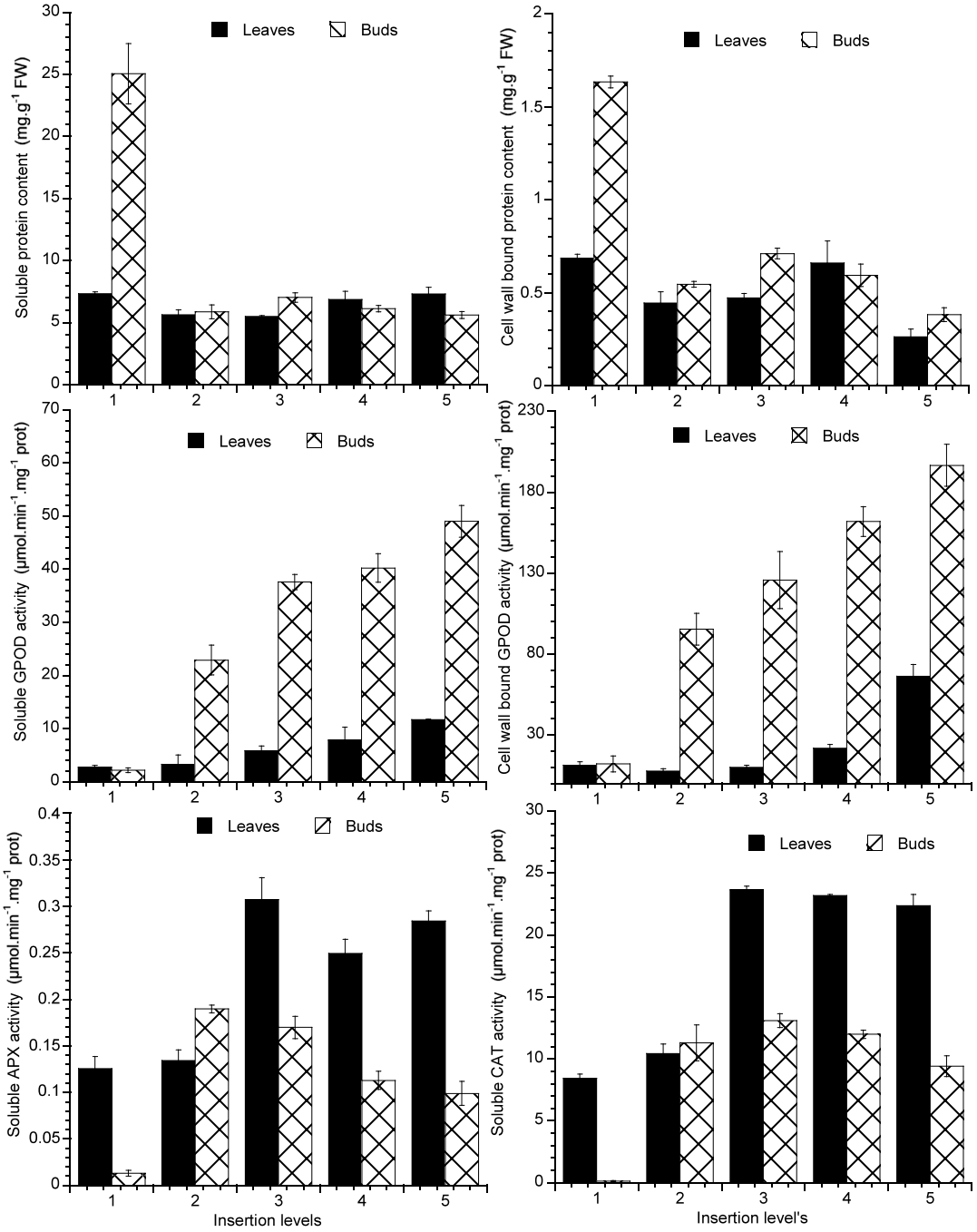

Fig. 4: Changes in protein (soluble and CWB) content and H_2O_2 scavenging enzyme specific activities in flower buds and leaves from shoot top (first insertion level, first node) to the fifth node below the apex (insertion level 5). Bars indicate the standard error (mean+/- S.E., n=5).

levels 3-5) whereas in buds, the activity was almost undetectable near the apex, it increased until node 3 and then decreased slightly although remaining at a comparable level until node 5.

Changes in protein content and enzyme activities in leaves of trees in which flowering was delayed by application of growth regulator

The effectiveness of growth retardants depends *inter alia* on their concentration and methods of application. Here, the lowest concentrations of retardants used delayed blooming by 14 d and 9 d for GA_3 and MH, respectively (Tab. 1). GA_3 and MH application delayed flowering without any further deleterious effects on photosynthetic capacity or fruit set (data not shown). When control trees were blooming (beginning of February) but not yet retardant-treated trees, protein contents in leaves were similar in all trees. However, G-POD, APX and CAT leaf specific activities were strongly lowered in treated trees. Higher concentrations of GA_3 and MH, and use of lemon cultivars other than "Eureka" also revealed a decrease in detoxifying enzyme activity associated with flowering retardation (not shown).

Discussion

Flower bud induction in lemon begins in November and continues until early January. Bud fattening starts to become evident late in January and full bloom occurs in late February [26 and Fig. 1]. In the present work, we followed H_2O_2 scavenging enzymes: G-POD, APX and CAT, first in leaves during the 24-week "cold" period preceding Full Bloom (from September to January), second in growing buds according to the developmental stage, and third in leaves and growing buds according to the insertion level along the length of the shoot.

CWB protein levels averaged 5% of soluble protein levels in leaves and buds of lemon twigs. These levels and the specific enzyme activities found in the present work (APX, G-POD and CAT, see all figures) were in the range of numerous studies previously reported for various plant organs and tissues, and referenced in the present paper. Due to the high G-POD specific enzyme activities in CWB proteins, cell walls appear to contribute to a significant fraction of H_2O_2 scavenging activity in leaves and flower buds in lemon (Fig. 3 and 4). This is in agreement with previous studies reporting high peroxidase activities in CWB protein fractions in plant tissues (Monerri and Guardiola, 2001; Liu and Lamport, 1974; Li et al., 1989; Lee and Lin, 1995; Kukavica et al., 2012). Nevertheless, the main objective of the study was to analyze the relative changes in biochemical parameters rather than to characterize their absolute values since artifacts can be caused during tissue extraction by retention of soluble proteins by the cell walls or membranes (Monerri and Guardiola, 2001). Due to the marked changes in leaf and bud specific enzyme activities (expressed per mg protein) which were several orders of magnitude higher than any changes in protein concentrations (expressed in mg of proteins per g of fresh weight), our data concerning specific enzyme activities can be compared to other data in the literature where activities were expressed on a mass basis.

From September to December (FB-24 to FB-10, Fig. 1), changes in specific G-POD, APX and CAT activities in leaves were small but the trends they presented occurred in the opposite direction to the changes in protein content (soluble and CWB). Thus, H_2O_2 scavenging enzyme capacity remained almost constant in leaves during the resting period. This could be linked to the fact that the trees were grown in a glasshouse without major environmental biotic and abiotic stresses which could have generated an oxidative burst. However, this also indicates that there was no oxidative stress in the leaves linked to the inductive effect of low winter temperatures on flower buds. However, leaf soluble and CWB specific G-POD activity was markedly increased (4-5 fold) in mid January (FB-5) as compared to the activity displayed from September to December. Mid January coincided with the end of bud dormancy and the beginning of growth (bud swelling). Interestingly, at this date, leaf APX activity was not maximal and leaf CAT activity was the lowest. Thus, considering the enhanced specific activity of H_2O_2 scavenging enzymes in leaves, only G-POD can be related to the beginning of flower formation. Bernal et al. (1993) also reported that an increase in peroxidase activity in leaves of *Capsicum annuum* accompanied flower formation.

The variations of G-POD, APX and CAT activity in both leaves and buds according to the position on the twigs in March are difficult to interpret (Fig. 4). This is due to the numerous factors which vary between insertion levels 1 and 5: stages of development for buds, age of leaves and environmental conditions (light), for example. Clearly, buds near the top of the twig were more numerous in earlier stages of development than buds located far from the apex. A marked gradient in specific G-POD activity (soluble and CWB) was observed along the shoots in leaves and buds (sampled buds were at developmental stage B for insertion level 1, C for insertion level 2, D for insertion levels 3 to 5), the activity being the lowest in the apical position (Fig 4). CAT and APX specific activities were also lowest near the shoot apex (nodes 1-2) but tended to decrease slightly from nodes 3 to 5. Taken together, these results indicate that only specific leaf and bud activity of G-POD increased with the developmental stage of the bud in relation with the position on the twigs.

During flower bud development (comparing buds sampled on the same node), G-POD, APX and CAT specific activities in buds increased from the beginning of bud swelling (stage A), peaked at full bloom (stage E) and then declined at fruit-set (Fig. 2 and 3). Changes in activity were remarkably similar for the three enzymes (including G-POD activity in CWB proteins). Meanwhile, changes

Tab. 1: Days to emergence of first flower buds, protein content, GPOD, APX and CAT specific activities in relation to growth regulator application.

	Days to emergence of first flower buds*	Protein content (mg · g^{-1} FW)		GPOD (µmol · min^{-1}.mg^{-1} prot)		APX (µmol · min^{-1}.mg^{-1} prot)	CAT (µmol · min^{-1}.mg^{-1} prot)
Treatment		soluble	CWB	soluble	CWB	soluble	soluble
Control	18.8a	7.08	0.44a	57.04c	30.21b	0.34b	29.99c
GA (50 ppm)	31.8c	7.75	0.62b	23.52b	27.79b	0.24b	16.77b
MH (1000 ppm)	26.7b	7.88	0.47a	10.16a	12.84a	0.13a	10.08a
LSD	1.73	NS	0.04	0.89	2.84	0.09	2.13

*days after treatment; LSD: Least Significant Difference, NS: not significant difference.

in soluble and CWB protein content showed the opposite trend. However, the magnitude of changes in enzyme activities was more than an order higher than protein contents. The burst of activities at FB was considerable since the values at this stage were 10- to 100-fold higher than in dormant buds. Enhancement of H_2O_2 scavenging enzyme activity from bud swelling and bud break and until FB may play a role in preventing any built-up of peroxide during the intense metabolic activity leading to flower formation. In plants, changes in CAT and POD activities related to bud development have already been described in several species. Data on APX activity in growing flower buds are scarce. However, it is noteworthy that APX activity is reported to decline in gladiolus following bloom (HOSSAIN, 2006) as observed here in lemon buds after FB.

KAMINSKI and ROM (1974) in peach, WANG et al. (1991) and ABASSI et al. (1998) in apple reported (very) low CAT activity in dormant flower buds and an increase in activity in the growing bud. In apple ABASSI et al. (1998), CAT activity continued to increase after FB to peak at fruit set and remained high for several days whereas in lemon buds, CAT declined strongly after FB (Fig. 3). Contradictory findings have been reported for CAT activity in dormant tissues. Indeed, as emphasized by ABASSI et al. (1998), NIR et al. (1986) and SCALABRELLI et al. (1991) observed that CAT in apricot, peach and grape was at its maximum during bud dormancy and minimal during bud break. Also, VITI and BARTOLINI (1998) in apricot flower bud indicated that CAT activity showed a marked tendency to decrease as bud fresh weight increased. They hypothesized, following SIMMONDS and SIMPSON (1972) and NIR et al. (1984), that an increase in peroxide content associated with a decrease in CAT activity can lead to the activation of the pentose phosphate pathway necessary for the resumption of active bud growth. Our results which clearly revealed an increase in CAT (and other H_2O_2 scavenging enzymes) in growing buds are not in agreement with this hypothesis.

CAT and POD activities were reported to increase simultaneously during leaf bud growth in apple (WANG et al., 1991). However, in the latter work, POD activity was relatively high in dormant buds, lower during the early stages of vegetative development, to increase again during bud growth. Also, evaluating POD in apple, ABASSI et al. (1998) observed high activity prior to bud break (swollen buds) which then declined during bud development until FB. ZAHRA et al. (2009) described decreasing POD activity from bud swell to FB in pistachio. They attributed the winter POD peak (December) to dormancy through oxidative stress caused by the cold. MONERRI and GUARDIOLA (2001) determining changes in ionically CWB and soluble POD in leaves and buds of flowering and non-flowering satsuma mandarin trees concluded that the POD activity was not a useful marker for the developmental stage of the buds in relation to flowering. This contrasts with the present results found in lemon which demonstrate that G-POD activity (soluble and CWB) in both leaves and flowering buds is strongly increased according to the floral stages of the bud from dormancy release (mid January) to FB (end of February).

CHANCE et al. (1979) estimated that approximately 2% of oxygen consumption during respiration results in H_2O_2 formation. It is possible that the increase in cell metabolism during flower development could have resulted from an accumulation of H_2O_2 due to higher rates of respiration. PODs can oxidize various phenolics, lignin precursors or secondary metabolites and are multi-functional enzymes involved in numerous physiological processes such as lignification, suberization, cross-linking of cell wall proteins, auxin degradation and control of apoplastic H_2O_2 levels (NORIEGA et al., 2007). Although PODs are normally involved in the dissipation of hydrogen peroxide, they also possess the ability to produce H_2O_2 via one-electron reduction of oxygen. H_2O_2 contributes to oxidative reactions in the apoplast both as a substrate in the process of polymerization and in cell wall loosening. It also contributes to defense reactions and signaling processes.

Taken together, our results reveal that G-POD activity (both soluble and CWB) in leaves and buds can be clearly related to flower development: in leaves at the end of the cold resting period, in buds according to the flower developmental stage (bud taken on a single node) and in buds and leaves according to the position along the twig which displayed a gradient in those stages. As underlined by MONERRI and GUARDIOLA (2001), using only one enzyme system to follow the transition from dormancy to floral state is a simple approach. However, in lemon, APX and CAT were also related to flower development although less clearly than G-POD. Application of substances to delay flowering without influencing either the photosynthetic capacity or the amount of proteins in leaves (thus without notably disturbing primary leaf metabolism) led to a marked decrease in specific activity of these enzymes in leaves. Thus, the changes in enzyme activities did not result from the direct influence of exogenous factors (such as light intensity, day length and temperature or water status) but appear to follow flower bud development. This strongly supports our conclusion of a close relationship between bud organogenesis during flowering and H_2O_2 metabolism independently of environmental factors. Because the depressive effect of flowering retardants on G-POD, APX and CAT was also observed in cultivars other than "Eureka" grown outdoors at another location in Tunisia with different climatic conditions, this suggests that the link between H_2O_2 scavenging enzymes and flowering is a general process in lemon.

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