Indoleacetic acid metabolism, phenols and growth in young kiwifruit berry

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Abstract

The patterns of auxin concentration and metabolism were investigated in distinct kiwifruit portions and compared with the rate of fruit growth during early developmental stages. Indoleacetic acid level was higher in inner fruit tissues, particularly in younger fruits, while the hormone was barely detectable in outer tissues. Modulation of free indoleacetic acid (IAA) concentration did not appear to depend tightly on conjugation of the hormone. Despite the lack of a strong correlation between the levels of IAA and IAA oxidase and peroxidases activities, the main enzymes in auxin catabolism, in some portions of the fruit a low hormone level corresponded to a higher IAA oxidase activity. An inverse correlation was also observed between hormone levels and the appearance/increase in some bands with high mobility in peroxidase gel activity assay. Phenols, compounds with a potential auxin-protecting activity, appeared to be more involved in photoprotection of the fruit than in the regulation of IAA levels. Beyond catabolism and conjugation, other metabolic pathways, particularly those occurring in the developing seeds, may have decisively influenced auxin levels in fruit tissues, as well as the amount of the hormone exported from the fruit. The latter, estimated by analyzing the concentration of IAA in the sap exuded from the pedicel, showed a time course which was similar to that displayed by inner fruit tissues. Furthermore, similarities were found between the pattern of IAA concentration in inner fruit tissues and fruit growth rate. The possible role of IAA in promoting growth during early fruit development is discussed.

Keywords: fruit growth, guaiacol peroxidase, IAA oxidase, indoleacetic acid, kiwifruit, phenols

Introduction

Most of chemical, structural and functional fruit traits change as fruit develops. In berries of kiwi (*Actinidia* spp), as in other fruits, the mineral composition, the vascular system and the structure of the skin greatly change during the growing season, in response to several internal and external factors (see Montanaro and others 2014 for review).

A series of physiological processes occurring during fruit growth affect fruit size at harvest, which is a key determinant of yield. Plant growth regulators widely affect those processes, being major endogenous signals for the control of fruit development (Gillaspy and others 1993; Davies 2010).

In several fleshy fruit species, auxin in combination with gibberellins and cytokinins play a major role in the regulation of fruit set, including the formation of seeds which in turn control cell division and fruit growth (Gillaspy and others 1993; Kumar and others 2014). Seed number is then positively correlated with fruit size and prolonged storage life, likely because it influences the accumulation of those nutrients involved in fruit structural stability (e.g. Ca) (Ferguson and others 1999; McPherson and others 2001; Brookfield and others 1996; Volz and others 1996; Howpage and others 2001). Fruit calcium accumulation depends, among others, on factors affecting the structure and function of the vascular system and the driving force of xylem stream, which greatly change during the early fruit developmental stage (i.e. 70-80 days after pollination, DAP) (Montanaro and others 2014).

Considering the relevance of xylem for fruit water balance and nutrient import (Montanaro and others 2015) and the involvement of auxin in certain steps of xylem formation (Sorce and others 2013), a detailed knowledge of seasonal changes of auxin concentration and metabolism is of great importance. It has been previously described the natural occurrence of auxin in young kiwifruit (Sorce and others 2011), however the hormone dynamics still await to be investigated in detail. Therefore this paper aimed at analyzing the time course of

indole-3-acetic acid (IAA) concentration and metabolism in kiwifruit, focusing on early fruit development, during which fruits considerably enlarge and almost attain their final size (Pratt and Reid 1974). The levels of both free and conjugated hormone molecules, as well as the activity of the major enzymes responsible for IAA catabolism, i.e. peroxidases and IAA oxidase (Arezki and others 2001; Normanly and others 2010) were determined. Based on the evidence that phenolic compounds may act as IAA-protectants (Krylov and Dunford 1996), potentially affecting IAA levels, this study also aimed at determining the concentration of phenols in order to further elucidate hormone metabolism.

It is known that the spatial distribution of minerals in fruit tissues is not uniform, with a higher concentration being found in the inner pericarp (including the seeds) than in either the outer pericarp or the pith (Faust and others 1969; Ferguson 1980; Clark and Smith 1991). For certain phloem-immobile nutrients, such a gradient could be related to an uneven development of the xylem, in response to auxin signals. Therefore, this study was designed to analyse IAA metabolism in distinct portions of the fruit along a longitudinal (proximal, median, distal) and a transversal gradient (inner and outer). The data of auxin metabolism were compared with those of fruit growth, to search for correlative evidence between hormone physiology and fruit development.

It has been proposed that a stream of IAA may regulate the transport of some cations (e.g. Ca^{2+}) in the opposite direction (Stahly and Benson 1970; Bangerth 1976; Banuelos and others 1987; Cutting and Bower 1989). Hence, polar auxin transport may be involved in this process. Given that part of polar auxin transport occurs outside the cells, it seems reasonable to assume that the concentration of apoplastic auxin may be an estimate of the potential magnitude of that transport (Bangerth 1976). Since the apoplastic IAA concentration has not been adequately investigated in kiwifruit, this study aimed also at evaluating the potential export of auxin from the fruit through the analysis of IAA concentration in the apoplastic sap artificially extruded from the fruit.

Materials and methods

Experimental site and fruit sampling

Trials were conducted at a commercial kiwifruit orchard (*Actinidia deliciosa* [A.Chev.] C.F. Liang et A.R. Ferguson cv. Hayward) in central Italy, as previously described (Sorce and others 2011), in the 2013 growing season. At bloom (May 18) approx. 400 fully-open flowers from the basal end of terminal fruiting shoots located in the central part of the canopy (1.5-2.5 m above the ground) were selected from 10 randomly chosen vines and hand pollinated using pollen previously collected from male flowers of 'Tomuri' vines growing in the same orchard. Approximately 30 uniform fruits were collected at 19, 27, 35, 45, 56 and 67 DAP from 6 shoots at each sampling date, sealed into plastic bags to minimize their transpiration and promptly transported to the laboratory. Twenty fruits were cut into three portions discarding the pedicel; the cutting lines were at 33% and 66% of berry length in order to obtain portions with similar thickness (Fig. 1); each portion was splitted in two sub-portions, namely 'inner' pericarp (including central columella and seeds) and 'outer' pericarp (including skin) (Fig. 1). These sub-portions were analyzed separately for IAA concentration, IAA catabolic enzyme activities and phenols. The remaining ~10 fruits were used for the collection of the sap (as described below).

Indoleacetic acid analysis in fruit tissues

Fruit sub-portions were chopped and dipped in cold 70 % (v/v) aqueous acetone (1:5, w/v). Three replications per sample were homogenized by mortar and pestle, divided into three subsamples and separately analyzed. Each replication (5-10 g FW) was processed according to the analytical protocol described in Sorce and others (2009). Briefly, samples were homogenized, supplemented with a suitable amount of $[^{13}C]_6$IAA as internal standard for

quantitative determination of endogenous IAA and extracted three times with aqueous acetone. The aqueous phase of the pooled extracts was partitioned against diethyl ether. The organic phase was dried, re-suspended in 0.5% (v/v) acetic acid and purified by C18 SPE cartridges. The fractions collected were evaporated, resuspended in the appropriate starting solvents and purified using HPLC. After diethyl ether partitioning, the aqueous phase was pooled with the extracted pellet and hydrolyzed. The first hydrolysis separated the ester-bound IAA and the second one separated the amide-bound IAA. At the end of these hydrolyses, samples were further purified by reverse phase HPLC. The HPLC fractions corresponding to the elution volume of the IAA standard were dried thoroughly, silylated and analyzed by gas chromatography-mass spectrometry. Mass spectra were acquired in full scan mode. Identification and quantification of the analyte were confirmed by tandem MS. Data presented for each sample are the mean of three replications ± SE and are expressed as ng g^{-1} FW.

Fruit sap collection and indoleacetic acid analysis

The potential export of auxin from the fruit during the studied developmental stage was evaluated by analyzing the concentration of free IAA in the apoplastic sap, collected by a Scholander pressure chamber using a procedure similar to that reported by Albacete and others (2008). For each sampling date ~10 fruits were used. Fruit pedicel was cut at the proximal end and inserted into a silicone tube passing through the rubber septum of the chamber. The standard pressure for all measurements was 1.3 MPa, to allow maximum flow rate of exuded sap according to Mazzeo and others (2013). Outside the chamber, the exuded sap was collected in 3 subsamples (2-5 ml each) from three different replications of 3-4 fruits each. Sap samples were stored at -20 °C until analysis. Just before IAA determination, samples were diluted to 5 ml with distilled water, adjusted to pH = 2.8 with HCl and supplemented with a suitable amount of $[^{13}C]_6$IAA as internal standard. Samples were

purified on C18 SPE cartridges, the volume of the fractions putatively containing IAA was reduced under low pressure, then the fractions were dried out under a nitrogen stream. Derivatization and GC-MS analysis of samples were performed as reported previously. Indoleacetic acid content was expressed as ng ml^{-1} sap.

Enzyme extraction and assay

Freeze dried fruit sub-portions were ground in an ice-cold mortar with liquid nitrogen and extracted in phosphate buffer (0.06 M, pH 6.1) containing PVP (1/1, w/w) and the homogenate was centrifuged (10000 x *g*, 5 min). The supernatant was recovered and used as a crude enzyme extract. Guaiacol peroxidase (POD, EC 1.11.1.7) activity was determined as described by Arezky and others (2001) using as substrate 1 % guaiacol. Enzymatic activity was determined following guaiacol oxidation by H_2O_2 (extinction coefficient 26.6 mM^{-1} cm^{-1}) at 470 nm, one unit oxidising 1.0 μmol guaiacol per min and was expressed as U g^{-1} protein. Indoleacetic acid oxidase (IAA oxidase, EC 1.13.16) activity was measured as described in Beffa and others (1990). The reaction solution contained 100 μM $MnCl_2$, 50 μM p-coumaric acid, 15 μg IAA and 200 μl of the extract in 1 ml 6.66 mM phosphate buffer pH = 6.0. After incubation at 24 °C for 40 min, 2 ml of modified Salkowski reagent (Pilet and Lavanchy 1969) were added. Enzyme activity was determined as IAA destruction at 535 nm after 30 min and expressed as U g^{-1} protein. One unit of IAA-oxidase activity is equivalent to 1 μg IAA oxidized by 1 ml of extract in 40 min. Protein measurement was performed according to Bradford (1976), using BSA as standard.

Electrophoretic peroxidase separation

Electrophoresis was performed on 10 % PAGE as in Milone and others (2003) with minor modifications. Tris-HCl 1.5 M pH 8.8 was used. Equal amounts (8.4 μg) of proteins extracted from the different fruit sections were loaded onto activity gel. After running

(200 V, constant current of 35 mA gel^{-1}) bands were visualized after incubation in the dark for 90 min in 1 M Na-acetate buffer pH 4.6 with 0.04 % benzidine and 10 mM H_2O_2. Enzyme activity appeared as dark brown bands. Incubation was also performed for 15 min in Na-acetate buffer pH 4.6 with 1 mM guaiacol and 10 mM H_2O_2.

In situ guaiacol peroxidase determination

Sub-portions of fresh fruits, at each sampling date, were sectioned using a cryostat. Cross sections (30 μm) were immediately immersed into a solution of colourless guaiacol/H_2O_2 (5 mM H_2O_2, 5 mM guaiacol in 60 mM phosphate buffer, pH 6.1). Peroxidase activity was revealed by dark/brown colour due to the conversion of guaiacol to tetraguaiacol. After 10 min of incubation the slices were washed three times in the same buffer and mounted in glycerol for the microscopy analysis (Lepeduš and others 2005 with minor modifications). A blank in the absence of H_2O_2 was made.

Extraction and determination of phenols

Total phenols were measured according to Arezki et al. (2001). Phenolic extracts were obtained after centrifugation of freeze dried fruit sub-portions homogenized in HCl 0.1 N and left at 20 °C for 3 h. Three hundred μl of extract were added to 1.5 ml H_2O + 0.1 ml Folin-Ciocalteu reagent and left so for 3 min. After addition of 400 μl Na_2CO_3 (20 % w/v) and incubation at 100 °C for 1 min, the samples were cooled in ice bath and the absorbance at 750 nm was measured. Phenolic compounds content were calculated as equivalent of gallic acid (GAE mg g^{-1} FW) on the base of a standard calibration curve.

Fruit growth

Fruit length (mm) was measured on 20 attached fruits randomly chosen from the vines used for fruit sampling (see above) at approx 10-day interval time from 0 to 100 DAP. Fruit

length values were used to calculate the relative growth rate (RGR) of the fruit over the period of two consecutive observations, and the average RGR values were referred to the mid-point of that period, according to Mazzeo and others (2013).

Statistical analysis

The data were the mean of at least three replicates from three independent experiments. Statistical significance was determined by ANOVA tests followed by *post hoc* Bonferroni multiple comparison test.

Results

Free and conjugated auxin concentration

The concentration of free IAA was significantly greater in the inner pericarp than in the outer one throughout the period studied, with the sole exception of the last sampling date (67 DAP), when similar values in inner and outer tissues were detected (Fig. 2, left column). The inner tissues showed an overall decline of the free IAA content, which was more pronounced in very young fruits (between 19 and 27 DAP). Notably, the initial value of IAA concentration was higher in inner tissues of M portion (approx. 55 ng g^{-1} FW) than in that of P and D (35 and 18 ng g^{-1} FW, respectively). The outer pericarp exhibited a similar course across the three transverse portions of the fruit, although the hormone concentration peaked at the last sampling date (67 DAP). Nevertheless, these peak values were comparable to the lowest ones that had been detected in the inner tissues. Among the fruit portions analyzed, the D one showed a lower hormone content when compared with M and P portions. In the outer tissues of D the concentration of IAA was even constantly below the detection limit.The time course of conjugated IAA concentrations displayed irregular patterns. Apparently, changes of both ester (Fig. 2, center column) and amide IAA (Fig. 2, right column) followed different patterns in comparison to that of the free hormone and, in most

cases, free IAA was more abundant than its conjugated forms. Remarkably, in M at the end of the experiment (67 DAP), amide IAA peaked in the inner tissues, whereas ester IAA peaked in the outer ones, attaining the greatest concentration value overall (approximately 200 ng g^{-1} FW; Fig. 2).

Apoplastic auxin

The level of free IAA detected in the exuded sap declined throughout the period of observation, starting from ~10 ng ml^{-1} on 19 DAP down to approximately 2 ng ml^{-1} on 67 DAP (Fig. 3). The decrease was more pronounced in very young fruits: between 19 and 27 DAP the concentration of the auxin underwent a 40 % reduction.

Peroxidase, IAA oxidase and phenols

Peroxidases and IAA oxidase activities are reported in Figure 4a and b, respectively. The activities of these enzymes were barely detectable in the outer pericarp: for this reason, no data are reported. The inner pericarp showed appreciable values for the activity of these enzymes, although changes of such activity yielded an irregular pattern, with increase of activity in different intermediate sampling times with maximum values of 110 (P and M portions) and 146 U g^{-1} (D portion).At the last sampling date, there was a general decline and activities were comparable (D) or lower (P and M) than those detected shortly after fruit set. Electrophoretic results relative to peroxidases in inner pericarp are shown in Figure 5. Only results from incubation in benzidine are reported, as the brown bands obtained from incubation in guaiacol began rapidly to fade after staining. Similar banding patterns were shown when benzidine and guaiacol were used as hydrogen donors. Staining revealed bands with lower and higher mobility, whose pattern changed depending on the portion considered. Noteworthy, the activity of bands with greater mobility increased during fruit

growth in proximal and distal portions, respectively (Fig. 5, P and D), while in the mid one these bands appeared only in late developmental stages, > 45 DAP (Fig. 5, M).

Initial concentrations of phenolic compounds were significantly higher in the outer tissues than in the inner ones, being 4.2-5.8 and 2-2.4 mg g^{-1} FW, respectively (Fig. 6). In the outer tissues the values were similar in the three fruit portions and declined to approximately 2 mg g^{-1} FW at the end of the experiment (Fig. 6). On the contrary, in the inner tissues phenol content had distinct patterns in the different fruit portions, reaching values, at the end of the considered period, that were similar to those detected in very young fruits (Fig. 6).

The *in situ* determination of POD activity evidenced a comparable pattern in fruits of different age. In particular, the activity was recorded in differentiating vascular bundles (Fig. 7b, c) and in the layer of cells delimitating the locule, i.e. the portion of inner fruit holding seeds (Fig. 7a). In early stages of fruit development (19 DAP), the area of hypostase showed a dark staining (Fig. 7d), while in later stages (56 DAP) peroxidases were localized at the seed coat (Fig. 7e, f).

Fruit growth

By the end of the period of study, the estimated average length of fruits was nearly 65 mm. The time course of fruit growth during the period under study yielded a nearly sigmoidal curve (Fig. 8). The RGR showed an initial peak of approximately 0.055 mm mm^{-1} day^{-1} (19-27 DAP), thereafter it progressively declined to the minimum value recorded at 80 DAP (Fig. 8).

Discussion

Free IAA showed a heterogeneous pattern in the three fruit portions. The concentration of auxin was higher in the inner part of the fruit, with a slight predominance of the M portion,

albeit such prevalence occurred only in younger fruits (i.e. around the first sampling date). The reason for this higher auxin amount in inner tissues is conceivably related to the presence in that fruit portion of the developing seeds, whose role as strong auxin source is well documented (Normanly and others 2010). Consistent with this data, the low level of auxin observed in the outer pericarp might be attributable to its spatial separation from the seeds and possibly to a scarce, if any, lateral translocation of IAA (either free, or conjugated) toward the peripheral region of the pericarp. This could be due to the limited development of radially-oriented vascular tissues in kiwifruit (Ferguson 1984). A similar radial gradient was observed in tomato fruits, where IAA was more abundant in the core region than in the peripheral one (Kojima 2005). Free-IAA concentration declined in all portions as fruit developed. At 45 DAP, when fruit had attained nearly 80 % of the final length and RGR had markedly reduced, the concentration of the hormone was around 10 ng g^{-1} FW in all portions. Experimental evidence on the seasonal pattern of IAA in kiwifruit berry is limited, hence it is difficult to discuss. Nevertheless, Li and others (2015) have recently found, in a closely related kiwifruit species (*A. chinensis*), a similar declining pattern of IAA, although the initial value, measured at fruit set (about 9 ng g^{-1} FW), was lower than that detected in this study. Similarly, IAA peaked in mango fruits shortly after fruit set, thereafter its concentration declined rapidly (Cutting and others 1986).

Hormonal activities has been related to nutritional aspects of plants. For example, IAA along with others factors (e.g. transpiration flux, nutrient demand, chemical properties of the conductive system) have been associated to Ca movement (Bangerth 1976; Banuelos and others 1987; Cutting and Bower 1989; McLaughlin and Wimmer 1999). Although transpiration is generally considered the dominant driver of Ca supply to various organs, informations are still limited to discriminate the relative importance of each factor. The free IAA of the fruit apoplast may be involved in a mutual relationship between polar basipetal auxin transport and acropetal Ca movement, as reported for tomato, apple and avocado

(Stahly and Benson 1970; Bangerth 1976; Banuelos and others 1987; Cutting and Bower, 1989). This mechanism could gain particular importance for Ca nutrition of fruits that grow under conditions that limit transpiration, i.e. that weaken the main driver of Ca supply (Montanaro and others 2010). Based on the evidence that the amount of free IAA of the fruit sap may be partly exported from the fruit (Kojima, 2005), some information could be inferred from our data. The time course of the apoplastic IAA appears to be related to that of the inner pericarp, which should be expected if the seeds are to be considered a major auxin source. Therefore, the data suggest that the changes of free IAA concentration that were detected in inner fruit tissues (changes putatively arising from the hormonal metabolism of the developing seeds) may have affected the amount of IAA potentially exported from the fruit.

Changes of the concentration of free IAA are the consequence of the differential regulation of hormone biosynthesis, conjugation, catabolism and transport (Woodward and Bartel 2005). Based on our results, it is difficult to envisage a role for conjugation in the modulation of free auxin levels. The time course of conjugated IAA concentration apparently was not related with that of the free hormone, thus confirming our previous observations in kiwifruit (Sorce and others 2011). Both ester and amide IAA displayed irregular patterns and their levels were rather low, with the exception of the peaks at 67 DAP, whose physiological relevance remains unknown. Notably, the peak of ester IAA in the outer M portion seems to be the origin of the peak of ester IAA that was detected in the whole fruits of the same age in a previous investigation (Sorce and others 2011). Although conjugation plays a critical role in auxin metabolism in the seeds of several species (Bialek and Cohen 1989a, b, Nowacki and Bandurski 1989), in kiwifruit the dynamics of conjugated IAA did not appear to significantly affect the level of the free hormone.

Enzymatic catabolism is generally considered to be a major regulator of the free auxin pool (Normanly and others 2010). In fact, the presence of POD and IAA oxidase was detected

only in inner tissues, where the content of IAA was generally higher in comparison with the outer parts of the fruit, underlining the possible involvement of these enzymes in the control of auxin content (Thomas and others 1982).

There was not a tight correlation between the trend of enzymatic activities and IAA content during the growth of kiwifruit. We can however remark that the distal portion of the fruit, which is characterized by a low IAA content, showed an overall higher IAA oxidase activity. Despite the lack of correlation between POD activity and IAA content, more detailed information could be gained from the analysis of the data obtained from the in-gel POD activity assay. Here, an inverse relation could be found between the activity of POD with higher mobility and the levels of free IAA. For example, mid fruit portions showed higher IAA content in younger fruits when these activity bands were absent, while the lower IAA content in late developmental stages coincided with the detection of such bands. Consequently, it could be suggested that the catabolic activities may give a significant contribution to the modulation of the concentration of free IAA. Furthermore, the study of peroxidase *in situ* may hint at other roles that this enzyme could play in kiwifruit growth. The localization of POD activity in correspondence of the vascular bundles was probably linked with the role of this enzyme in the process of lignin biosynthesis (Lee and others 2007), a fundamental step of xylem formation. A similar role could explain the presence, at early developmental stages, of peroxidases in correspondence of the seed hypostase, which is constituted by cells with lignified walls (Marzinek and Mourão 2003), and also in seed teguments during the late stages of growth. The POD activity detected in correspondence of the seed locules could contribute both to the control of the concentration of the IAA produced by the seeds and to the protection of these organs from reactive oxygen species, that are key components of seed biology (Bailly 2004).

Phenolic compounds are important secondary metabolites (Kefeli and others 2003), involved in various physiological processes of fruit growth and development (Cheniany and others

2010) and are also known as protective agents against peroxidase-induced IAA oxidation (Krylov and Dunford 1996, Montanaro and others 2006). These compounds are defensive factors under biotic and abiotic stress conditions (Solar and others 2006), playing an important role in protection from UV radiation (Lattanzio and others 2006; Huyskens-Keil and others 2007) and, accordingly, their content was shown to increase in kiwifruit under high irradiance during early maturation (Montanaro and others 2006). This important shielding action was in agreement with the higher concentration of phenols recorded around 19-27 DAP in the outer tissues of the fruit, where the level of irradiance could have stimulated the biosynthesis of these photoprotecting molecules. In later stages (~40 DAP) the outer layers of the fruit form a periderm, with suberized cell walls (Montanaro and others 2014). This tissue could represent a more effective defence against high irradiance, thus lowering the importance of phenols, whose content stood on lower values, similar to those recorded in the inner portions of the fruit. The eventual action of phenols as IAA-antioxidants could be played only in the inner portions of fruits, where POD activity was recorded.

Initial fruit growth occurs by cell division (Hopping 1976): by 67 DAP the average length of fruits had attained a value that corresponded to 85 % of the average length of the product at harvest maturity (data not shown). The time course of fruit elongation was markedly similar to that of free IAA concentration in the inner fruit tissues. Auxin may enhance the sink strength of an organ, such as a fruit, by increasing cell number, cell size and by regulating cell differentiation (including xylem differentiation; Varga and Bruinsma 1976, Brenner and Cheikh 1995). According to this evidence, a role may be envisaged for IAA in the stimulation of kiwifruit growth during this early developmental stage.

In conclusion, auxin might operate positively in regulating the development of kiwifruit berries: besides the direct stimulation of tissue growth, IAA would induce the differentiation of new xylem vessels, thus putting forward this growth regulator as a key factor in the

control of early development. The concentration of the free hormone is under the control of several mechanisms, among which biosynthesis (probably in the developing seeds) and enzymatic catabolism seem to play a primary role, although their importance may change, depending on the fruit tissue and the developmental stage. Phenols seem to be less involved in the regulation of IAA levels, while they could play a significant role in photoprotection.

The present work may contribute to foster our understanding of the physiological implications of auxin changes for fruit development in kiwi and represents a starting point for further investigations on this matter.

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505 **Figure legends**

506

507 Fig. 1

508 Schematic representation of (left) proximal (P), median (M), distal (D) portions of fruit and
509 (right) "inner" and "outer" sub-portions used for the analysis of IAA concentration, IAA
510 catabolic enzyme activities and phenolic compounds.

511

512 Fig. 2

513 Changes of free (left), ester conjugated (center) and amide conjugated (right) IAA
514 concentrations in inner and outer pericarp tissues of different fruit portions (D = distal; M =
515 median; P = proximal fruit portion) during the experiment. DAP = days after pollination.
516 Asterisks indicate values that are significantly different, at each date, between inner and
517 outer tissues ($p < 0.05$). The results represent the mean of three replications ± SD.

518

519 Fig. 3

520 Concentration of free IAA in the sap collected from the cut pedicel of the fruit during the
521 stage of cell division growth. DAP = days after pollination. The results represent the mean
522 of three replications ± SD.

523

524 Fig. 4

525 (a) Guaiacol peroxidase (POD) and (b) IAA-oxidase activities in proximal (P), middle (M)
526 and distal (D) inner portions of fruits during the stage of cell division growth. Enzymatic
527 activity is reported as U g^{-1} protein. DAP = days after pollination. The results represent the
528 mean of three replications ± SD.

529

530 Fig. 5

Native PAGE gels of guaiacol peroxidase in proximal (P), middle (M) and distal (D) inner portions of fruits during the experiment. DAP = days after pollination.

Fig. 6

Phenol content in inner and outer pericarp tissues of different fruit portions (P, proximal; M, middle; D, distal), during the experiment. Phenol content is reported as mg gallic acid equivalent (GAE) g^{-1} FW. DAP = days after pollination. The results represent the mean of three replications ± SD.

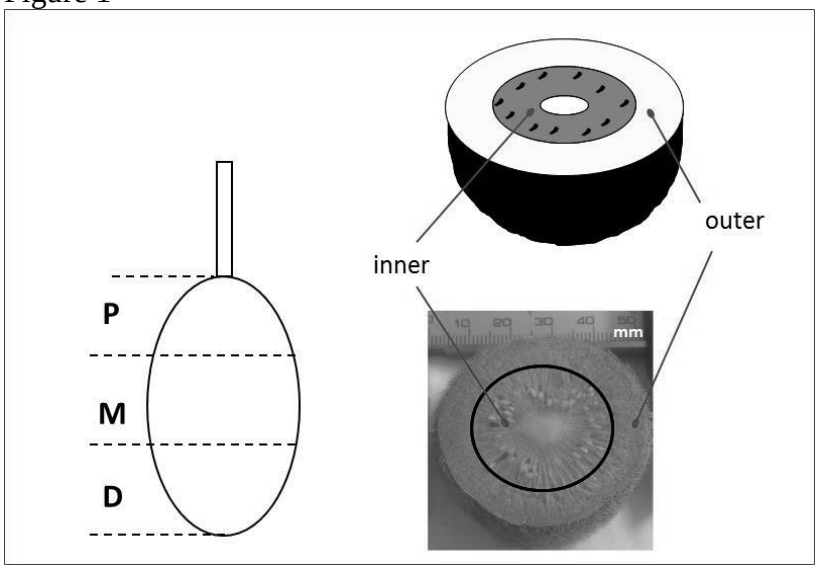
Fig. 7

Cross sections of inner pericarp tissues of different fruit portions for *in situ* determination of guaiacol peroxidase activity. (a) Cells delimitating the locule (19 DAP, proximal portion), (b, c) vascular bundles (19 and 56 DAP respectively, middle portion), (d) hypostase (19 DAP, middle portion), (e) seed and (f) particular of seed coat (56 DAP, middle portion). DAP = days after pollination. Bars indicate 200 μm.

Fig. 8

Fruit length (dashed line) and relative growth rate (RGR, solid line) of the fruits collected from full bloom to 100 days after pollination (DAP). Each value represents the mean ± SD of 20 fruits. Values of RGR were plotted at the middle of each observation period.

552 Figure 1



553
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555 Figure 2

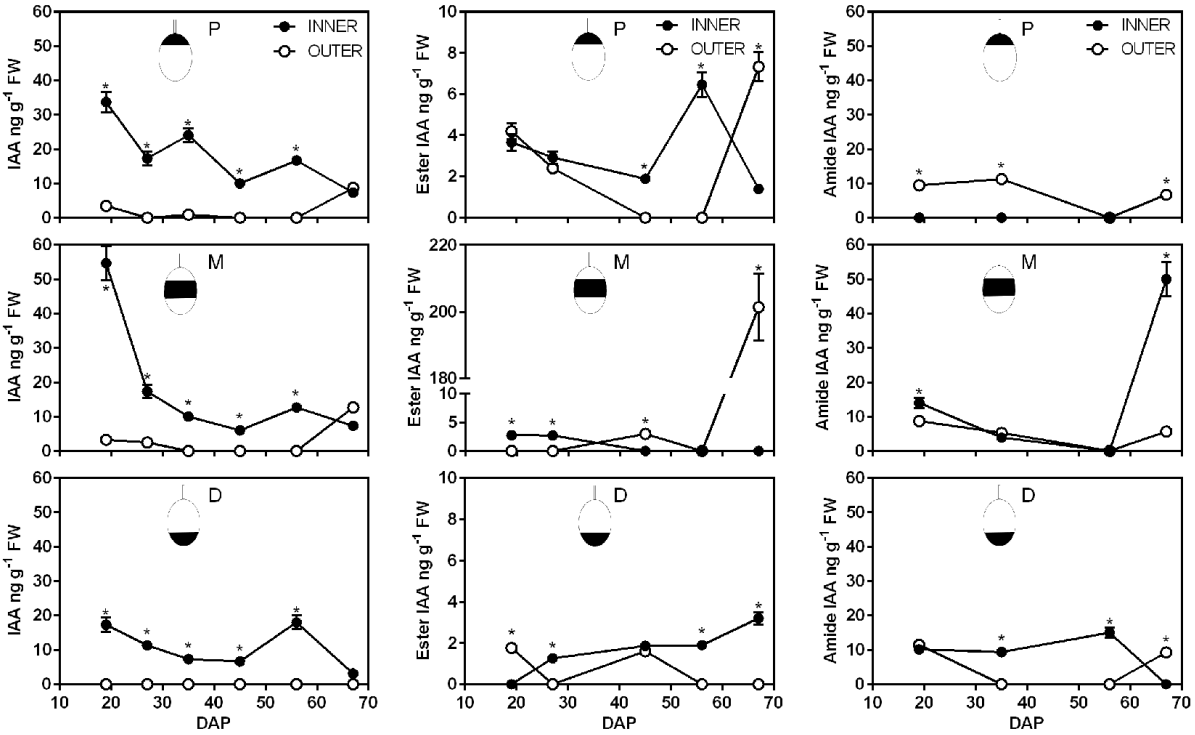

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Figure 3

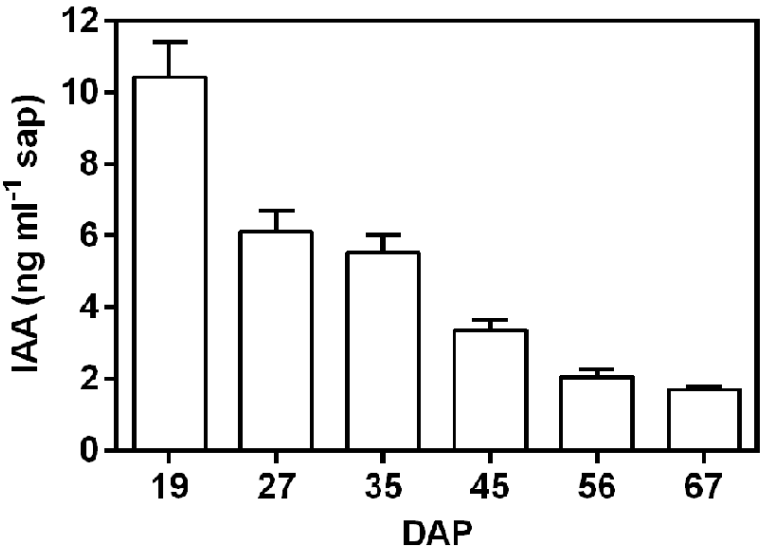


Figure 4

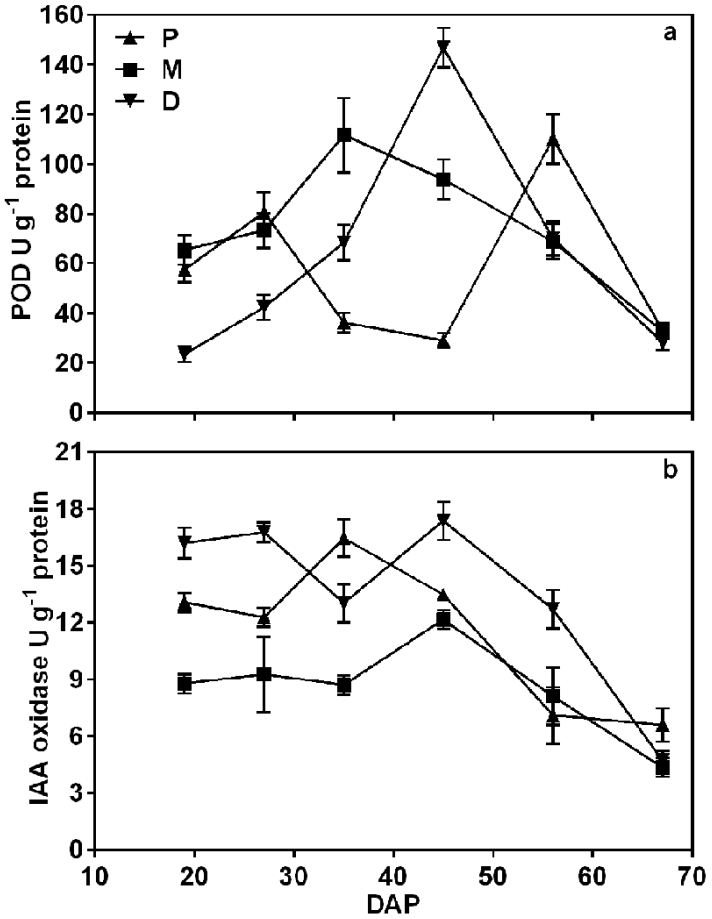


Figure 5

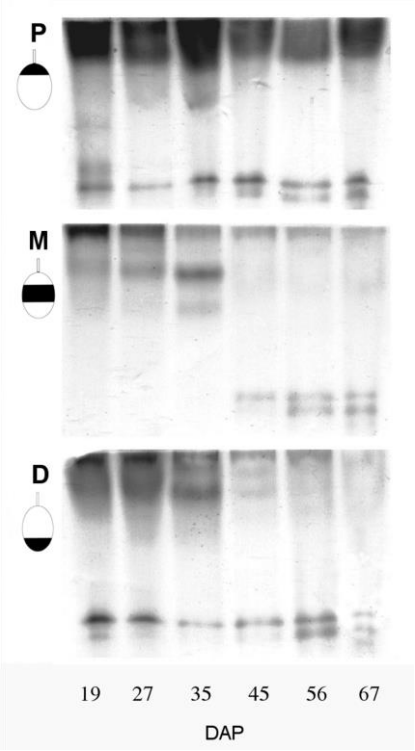
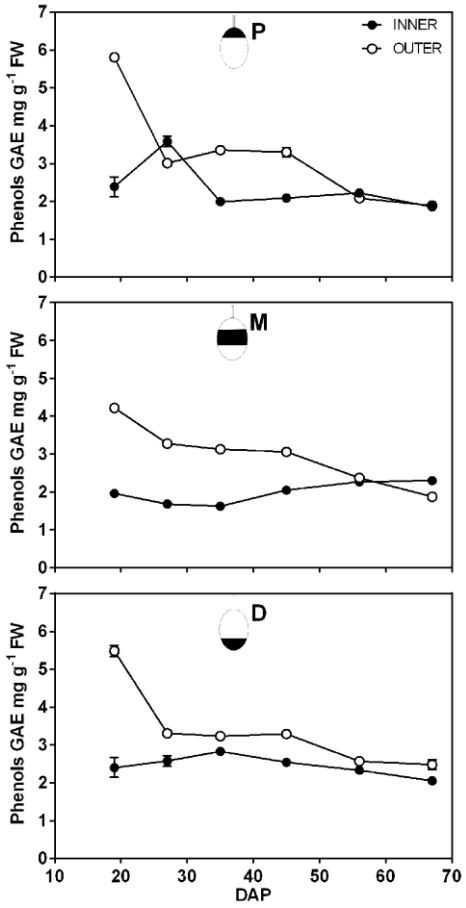
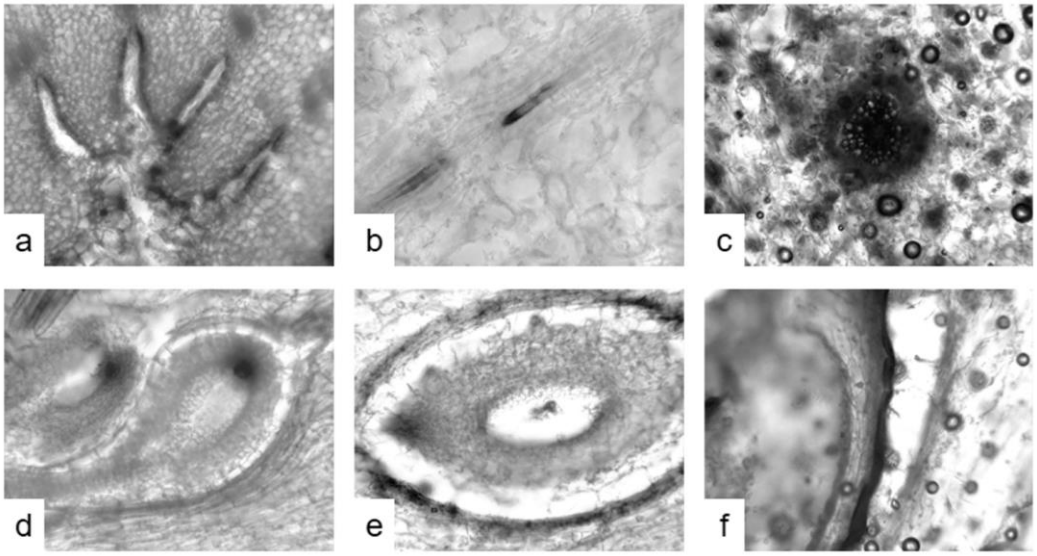


Figure 6



584 Figure 7



585
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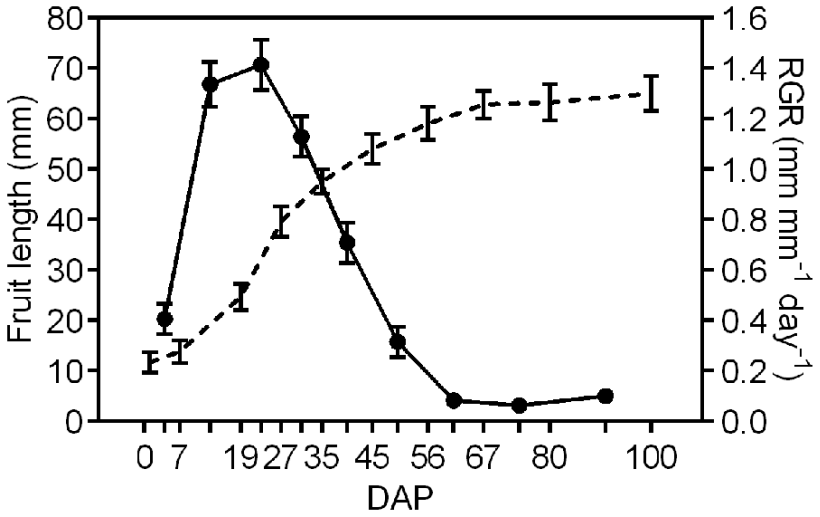
587 Figure 8



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