


Ethylene sensitivity regulates the wounding response in wild type and never ripe tomatoes

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Abstract

Tomatoes are important sources of vitamins, minerals, and bioactive molecules. The fresh cut industry is interested in including tomatoes among the fresh cut vegetables. The wounds usually induce tissue degradation and release of juice, which can negatively affect quality during storage. The aim of this work was to investigate the role of ethylene in the processed vegetables using the never ripe (Nr) mutant and wild type tomatoes. The ethylene accumulation affected shelf life and quality based on the differential sensitivity of the genotypes to ethylene. Physiological and biochemical parameters related to membrane stability, phospholipases activities, ethylene and carbon dioxide accumulation were investigated in Nr mutant and wild type tomatoes as processed products and whole fruits. Results indicate that ethylene biosynthesis significantly regulates membrane breakdown. Nr tomatoes showed higher membrane stability, higher tolerance to wounding, and lower variability of physiological and biochemical parameters. Hence, this genotype can represent a genetic source of traits that can be exploited in fresh-cut tomato breeding programmes.

Keywords: CO₂; electrolyte leakage; fresh-cut; lipid peroxidation; membrane stability; Nr mutant; storage

Introduction

The fresh-cut vegetables market sharply increased in the last two decades in most developed countries and tomatoes have been increasing as a component of ready to eat meals (Ahmed, Martin-Diana, Rico, & Barry-Ryan, 2012; Nassivera & Sillani, 2015).

Fresh-cut produce after processing undergo deep physiological, biochemical, and transcriptome changes in response to slicing or wounding (Artés, Gómez, & Artés-Hernández, 2007; Baldassarre et al., 2015). In wounded tissues, ethylene biosynthesis usually increases as well as its accumulation in storage boxes or bags (Cavauiolo et al., 2015). Ethylene can act as trigger of senescence and

postharvest disorders that compromise the quality of fresh cut produce (Watada & Qi, 1999). However, the negative ethylene effect depends on tissue sensitivity, rather than biosynthesis (Blankenship & Dole, 2003). To avoid the negative ethylene effects in fresh-cut tomatoes, products are preserved using modified atmosphere packaging. Usually the high carbon dioxide and lower oxygen concentrations inhibit the last step of ethylene biosynthesis catalysed by 1-aminocyclopropane-1-carboxylate (ACC) oxidase. The reduced ethylene biosynthesis also decreases the accumulation of this hormone inside the packaging or storage containers (Aguayo, Escalona, & Artés, 2004). In fresh-cut tomatoes, high ethylene reduced the development of water soaking areas, because of the beneficial action of the hormone against the incidence of chilling injury (Hong & Gross, 2000, 2002). In contrast, Jeong, Brecht, Huber, and Sargent (2004) found that the ethylene action inhibitor, 1-methylcyclopropene (1-MCP), reduced the development of water soaking and this phenomenon was not related to storage temperature. The 1-MCP reduces ethylene sensitivity blocking the ethylene receptor. The same behaviour can be observed in a constitutive ethylene insensitivity found in never ripe (Nr) tomato, which carries a mutation that blocks ethylene perception (Lanahan, Yen, Giovannoni, & Klee, 1994). This mutant can be useful for understanding the role of ethylene insensitivity in wound responses.

In fact, in fresh-cut produce, slicing hastened tissue deterioration and induced softening that is considered the major limiting factor that compromises fresh-cut tomato quality (Pinheiro & Almeida, 2008). Loss of texture is related to cell wall disassembly and turgor pressure reduction (Pinheiro & Almeida, 2008; Saladié et al., 2007). Loss of turgor pressure, instead, depends on cell membrane integrity. The damage of cell membrane leads to an increase in membrane permeability that can be measured by electrolyte leakage or lipid peroxidation (Natalini, Martinez-Diaz, Ferrante, & Pardossi, 2014). Membrane breakdown is considered to be a consequence of physical alteration in the double layer of the cell membrane, as it can be observed in chilled, injured tissues (Saltveit, 2002), and by the activity of reactive oxygen species (ROSs or membrane hydrolytic enzymes such as phospholipases [PLC, EC 3.1.4.3; PLD, EC 3.1.4.4]) (Antunes & Sfakiotakis, 2008; Boukobza, Dunphy, & Taylor, 2001; Wang et al., 2000). Pinheiro, Almquist, Novotna, and Paliyath (2003) demonstrated that PLD suppression can help in maintaining tissue integrity and reduced the loss in colour of fresh-cut tomato. In sliced tomato, the PLC and PLD activities were strictly correlated with membrane integrity indicators such as electrolyte leakage or lipid peroxidation (Natalini et al., 2014). Interestingly, Pinheiro et al. (2003) suggested the potential role of PLD in ethylene signal transduction with assumed distinct functions in molecular signal transduction for ethylene receptors SIETR-1 and SIETR-2 (Whitelaw et al., 2002).

The aim of this work was to investigate the effect of ethylene sensitivity on membrane deterioration in intact or sliced tomato fruit. The study was carried out using two genotypes of tomato: wild type (wt, as control) and never ripe (Nr mutant), which is insensitive to ethylene action. In both genotypes, membrane integrity as well as the role of phospholipases were also studied.

Materials and methods

Plant material and growing conditions

Tomato plants (*Solanum lycopersicum* L.) cv. Gimar wt (nr/nr) and its Nr isogenic mutant (Nr/Nr) were hydroponically grown in a heated glasshouse (minimum temperature 10°C) in Pisa (latitude 43°43'N; longitude 10°23'E; Italy).

Plants were grown in a nutrient film technique (NFT) system with a plant density of 3 plants m⁻². Drip irrigation was conducted by using a nutrient solution with electrical conductivity (EC) 3.5 dS m⁻¹ and pH 6.5. The recirculating nutrient solution was discharged after 3 weeks or when the electrical conductivity (EC) was higher than 6 dS m⁻¹.

Plants were tagged at anthesis (defined as the time of petal drop and fruit set). Tomatoes were harvested on the second cluster of 4 plants per each genotype a single day at c.a. 50 days after anthesis corresponding to the pink colour stage and pink-like stage for Wt and Nr mutant, respectively. Fruits were also selected according to their morphological aspects: colour, shape, and size.

Whole fruits were dipped into cold tap water, immersed for c.a. 1 minute in sodium hypochlorite solution (1 mg L⁻¹), rinsed in tap water and then dried with paper tissue. Four to five 10 mm thick slices were cut from the proximal end portion of the fruit with a stainless-steel knife and the outermost slices were discharged. Intact tomatoes (as control) and the equatorial slice of cut fruits were selected and placed into 1.1 L plastic (polyethylene), gas tight containers. Storage was conducted at 4.0 ± 0.5 °C in dark conditions.

Electrolyte leakage determination

Electrolyte leakage was assessed according to Saltveit (2002) with slight modifications. At each sampling point, four freshly excised cubes of about 1 g FW were collected from the outer pericarp of each slice. Samples were gently rinsed into approximately 200 mL of an isotonic 0.4 M mannitol solution to remove juice and liquid (due to cutting), in order to avoid interferences with initial electrolyte leakage readings. The cubes were blotted dry, placed into 20 mL of 0.4 M mannitol

solution and incubated for 3 h at room temperature with slow shaking. Immediately after incubation, conductivity was measured by using a conductance meter to obtain the initial value of EC. The cubes of pericarp tissues were separated from the solution (stored at 4 ± 0.5 °C) and frozen overnight (o/n) to obtain complete cellular disruption. Total electrolytes were also determined on the same sample after being frozen at -20 ± 1.0 °C o/n and thawed at room temperature. Electrolyte leakage was expressed as a percentage of the total electrolyte readings.

Lipid peroxidation determination

Lipid peroxidation was assessed by the protocol described by Health and Packer (1968) and subsequent modifications. About 3 g FW were taken from the outer pericarp of each sample, frozen in N₂ and stored at -80 °C until analysis. Each fruit sample was homogenised in 1.15 mL buffer solution containing 0.1% trichloroacetic acid (TCA) and centrifuged at $15,000 \times g$ for 10 min in a refrigerated centrifuge at 4°C. To a 1 mL aliquot of supernatant, 4 mL of 20% TCA and 250 µL 0.5% thiobarbituric acid (TBA) were added. The reaction mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. Then, the tube was centrifuged at $15,000 \times g$ at 4 °C for 10 min and the absorbance of the supernatant was read at λ_{532} and λ_{600} nm. The value for the non-specific absorption at λ_{600} nm was subtracted from the λ_{532} nm reading. The concentration of thiobarbituric acid-reactive substances (TBARS) was calculated using the extinction coefficient of $155 \text{ mmol}^{-1} \text{ cm}^{-1}$ (Health & Packer, 1968). TBARS content was expressed on a sample fresh weight basis ($\text{nmol g}^{-1} \text{ FW}$).

Ethylene and CO₂ evolution

Ethylene and CO₂ concentrations were measured by enclosing fruits samples in plastic containers (1.1 L). Intact fruits were used as control or sliced tomato (as fresh-cut treatment). To analyse the levels of accumulated ethylene or CO₂, gas samples (2 mL each) were taken from the headspace of the storage containers with a hypodermic syringe after 24, 48, 72, 120, 144, and 168 h of storage at 4 °C. The ethylene and CO₂ concentrations in the container were measured by gas chromatography (HP5890, HewlettPackard, MenloPark, CA, USA) using a flame ionisation detector (FID), a stainless steel column (150×0.4 cm packed with Hysep T). Column and detector temperatures were 70 and 350 °C respectively, and nitrogen carrier gas was used at a flow rate of 30 mL min^{-1} . Results were expressed as absolute content ($\mu\text{L L}^{-1}$) and per normalised content on fresh weight basis ($\text{pL ml}^{-1} \text{ g}^{-1} \text{ FW}$ and $\mu\text{L L}^{-1} \text{ g}^{-1} \text{ FW}$) for ethylene and CO₂, respectively.

PLC and PLD enzymes extraction and assay

PLC and PLD activities were assayed according to the protocol described by Mao, Karakurt, and Huber (2004) with some modifications. About 6 g FW was taken from the outer pericarp of each sample, frozen in N₂ and stored at -80 °C until analysis.

Briefly, tomato pericarp tissue was ground to a fine powder in liquid N₂ with a mortar and pestle. Each 1 g of the homogenate was mixed with 1 mL of 50 mM Tris-HCl (pH 8.0) containing 10 mM KCl, 500 mM sucrose and 0.5 mM phenylmethylsulfonylfluoride (PMSF). After centrifugation at 15,000 × g at 4 °C for 30 min, the supernatant was used for PLC and PLD activities by using p-nitrophenylphosphorylcholine (NPPC, Fluka BioChemica – Sigma-Aldrich, Italy) as substrate.

Reaction mixtures for PLC consisted of 1.0 mL of 0.25 M Tris-HCl (pH 7.2) with 20 mM NPPC 60% -sorbitol and 0.3 mL (about 300 µg protein) of the cell-free protein extracts.

For PLD, 0.9 mL of 50 mM Ca-acetate, pH 5.6 containing 27.4 mM NPPC was mixed with 0.1 mL (0.4 units) of acid phosphatase (Sigma-Aldrich, Italy) dissolved in 50 mM Ca-acetate (pH 5.6) along with 0.3 mL of the cell-free protein extract. After 60 min incubations at 37 °C, 0.1 mL of 50 mM NaOH was added and p-nitrophenol content was determined at 400 nm. Protein content was measured using the Biorad protein assay method (Biorad, Munchen, D, UE) with bovine serum albumin (Sigma) as standard (Bradford, 1976). Activities of PLC and PLD were expressed as $\mu\text{Kat mg}^{-1}$ protein.

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) separately per each storage time, considering as variable the two genotypes and wounding effect (control versus sliced). Differences among means were determined by the least significant difference (LSD) multiple range test ($P < 0.05$). Linear regression and correlations among measured parameters were performed.

Results

Electrolyte leakage and lipid peroxidation

Membrane integrity in both genotypes was evaluated by measuring the electrolyte leakage or TBARS content. Electrolyte leakage in intact fruits did not change during storage. In the Nr mutant, the electrolyte leakage values were around 10% (Figure 1(a)), while in wt intact fruit the values were around 18%. The electrolyte leakage in sliced tomatoes, in both genotypes, did not increase in the 24 h after slicing, while after 72 h the increase was an average of 10%, reaching 20% in Nr sliced

fruits and 31% in wt sliced fruit (Figure 1). The electrolyte leakage remained stable from 72 h until the end of experiment, while wt sliced fruits showed a further increase after 168 h of storage (Figure 1(a)).

Lipid peroxidation assessed using TBARS assay method showed almost the same trend as electrolyte

leakage. In intact fruits, TBARS did not significantly increase during the experimental period. In sliced fruits, instead, the TBARS increased (Figure 1(b)). The arise of TBARS in Nr sliced fruits was observed earlier, after 72 h, while in wt the sharp increase was observed after 120 h. In both sliced fruits, the TBARS declined at the end of the storage period (Figure 1(b)).

Absolute and normalised ethylene and carbon dioxide

The ethylene content (absolute and normalised) in storage boxes with both intact and sliced tomatoes of the two genotypes continuously increased during storage at 4 °C (Figure 2(a)–(b)). The ethylene increased after 24 h in wt tomatoes both intact and sliced. In wt sliced fruits the ethylene was constantly higher than other treatments, reaching 41.1 $\mu\text{L L}^{-1}$ or 335.9 $\rho\text{L mL}^{-1} \text{g}^{-1} \text{FW}$ at the end of the storage.

The levels of absolute ethylene inside the boxes at 168 h of storage were 23, 17, 27, and 47 folds higher compared with levels at time 0 for Nr intact, Nr slice, wt intact, and wt sliced, respectively (Figure 2(a)). The ethylene normalised, instead, for intact and sliced fruits of wt and Nr were 17, 9, 50, and 15 folds higher, respectively, compared with values at time 0 (Figure 2(b)).

The CO₂ levels slowly increased during storage in boxes containing intact or sliced tomatoes in both genotypes for absolute and normalised content (Figure 3(a)–(b)). The CO₂ concentration was higher in wt sliced tomato at the end of storage. In particular, CO₂ content in the boxes were 2, 4, 5, and 7 folds higher compared to the time 0 for wt sliced, Nr intact, Nr sliced, and wt intact, respectively. A similar pattern was observed for normalised CO₂ content. At the end of storage, normalised CO₂ concentration in boxes containing intact or sliced fruits of wt and Nr were 4, 2, 2, and 3 fold higher, respectively, compared to the initial values for Nr sliced, wt intact, Nr intact, and wt slice, respectively.

Phospholipase C and D activities

The PLC activity in intact tomatoes of both genotypes increased after 120 h of storage (Figure 4(a)). In sliced fruits, the PLC increased earlier in Nr tomato and values were constantly higher than other

treatments during the entire experimental period, except for the last time point that showed similar values of wt sliced fruits. In sliced wt fruits, the PLC activity increased at 72 h of storage (Figure 4(a)).

The PLD activity remained unchanged until 72 h of storage without differences among treatments and genotypes (Figure 4(b)). The PLD activity increased earlier in wt sliced tomatoes than in Nr sliced tomatoes. The PLD enzyme activity increased after 120 h of storage in wt sliced fruits and at the end of the experiment in Nr sliced tomatoes. At the end of storage, the PLD increases were 3 and 4 fold higher than the initial value in sliced wt and Nr genotypes. In intact tomatoes of both genotypes, the PLD activity did not change during the whole experimental period (Figure 4(b)).

Correlation analysis between electrolyte leakage and TBARS content (Figure 5(a)), electrolyte leakage and PLD activity (Figure 5(b)), TBARS content and PLD activity (Figure 5(c)) were calculated. All the measured parameters showed significant correlations. On the contrary, PLC activity was not correlated, neither to electrolyte leakage nor to TBARS content. The activities of both PLD and PLC enzymes showed a moderate degree of correlation at high significance level ($r = 0.48$ at $P < 0.001$).

Discussion

The Nr, an ethylene mutant tomato genotype, is a useful tool for studying the role of ethylene under different abiotic stresses (Di Baccio et al., 2012). Electrolyte leakage is commonly accepted as a measure of membrane integrity to appreciate the extent of damages related to chilling injury or to cutting (Jeong et al., 2004). Similarly, TBARS content is commonly used for evaluating the lipid peroxidation of cell membrane as a result of reactive oxygen species damage. In tomatoes, fatty acids of cell membranes are mainly represented by linoleic and linolenic acids (Fu & Huang, 2001). Data obtained from electrolyte leakage and lipid peroxidation indicated that Nr tomatoes have stable membranes compared to the wild type genotype. According to the literature, this result can be associated with lower tissues ethylene sensitivity in Nr tomatoes. Moreover, no ethylene burst was observed in the mutant, demonstrating that the system 2-autocatalytic ethylene production is inhibited due to the inability of the mutant Nr receptor to bind ethylene (Hackett et al., 2000; Lanahan et al., 1994). Our data, regarding membrane stability and enzyme activities, in sliced and intact tomatoes of wt genotype confirm those observed in earlier reports carried out on s tomatoes stored at chilling temperatures (Aguayo et al., 2004; Gil, Conesa, & Artés, 2002; Hong & Gross, 2000, 2002; Natalini et al., 2014).

In the wt genotype, the higher ethylene concentration is presumably the result of a wound-induced response, as also suggested by Hong et al. (2000) for an ethylene sensitive tomato genotype. Also the CO₂ levels in fresh-cut boxes gradually increased during storage. CO₂ levels for Nr intact fruits were lower compared to Nr sliced fruits. Presumably, wounding increased respiration in the Nr genotype, whilst no stimulation occurred for wt tomato slices compared to wt intact fruits. Watada and Qi (1999) also found similar respiration rates in intact and sliced tomatoes in an ethylene sensitive genotype.

The PLC activity seems to be activated since the Nr tomatoes promptly enhanced the enzyme activity. The PLD instead followed the same trend of the wt sliced tomatoes but the increase was delayed and an ethylene regulation may be suspected. Interestingly, PLC and PLD activities were strictly correlated at high significance level ($r = 0.81$ at $P \leq 0.001$, data not shown). This finding is consistent with the working model on phospholipid catabolism proposed by Wang et al. (2000) for Arabidopsis leaves. This result confirms the importance of PLD in the wounding response in fruits, by promoting phospholipids degradation that also involves PLC activity (Wang et al., 2000).

It is likely that phospholipases activate downstream lipid catabolism leading to membrane peroxidation and loss of membrane integrity assessed by means of TBARS content and electrolyte leakage. Anyway, it is possible that the degradation of membrane lipids (e.g. glycolipids) other than phospholipids could be involved by providing fatty acids prone to be peroxidated with subsequent increase in electrolyte leakage. Thus, lipid peroxidation and subsequent membrane leakage are not only related to phospholipid degradation, but depend upon overall membrane lipid catabolism.

Interestingly, TBARS content and electrolyte leakage were strictly correlated with sliced fruits and in particular in the wt genotype. These finding suggest that electrolyte leakage, as well as lipid peroxidation, are two suitable parameters that can be used to detect wounding damage in tomato tissues. These results are in agreement with our previous study carried out with cv. Jama stored at 4 °C for 168 h after slicing

(Natalini et al., 2014).

Combined results for both wt and Nr genotypes suggest that slicing induces a significant increase in electrolyte leakage, lipid peroxidation and PLD activity, as well as ethylene accumulation inside the container. However, Nr genotypes show a reduced amplitude of variation of the indexes by slicing. This behaviour can be ascribed to lower ethylene biosynthesis and/or impaired ethylene perception.

The findings of the present work for Nr mutant showed that loss in membrane integrity and the increase of lipid peroxidation and in phospholipase activities are due to wounding and ethylene tissue sensitivity rather than ethylene biosynthesis or accumulation inside the storage boxes.

Ethylene sensitivity reduces and delays the tissue and cell membrane damage after wounding response, but it does not modify the pattern of response.

In conclusion, our results suggest that lower ethylene production significantly regulates the membrane breakdown, involving lipid peroxidation, electrolyte leakage and PLD enzyme activity. Nr tomatoes have higher membrane stability and higher tolerance to wounding, exhibiting lower variability of physiological and biochemical parameters. Therefore, this genotype can represent a genetic source of traits that can be used in the fresh-cut tomato breeding programmes.

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All authors contributed equally to this work.

Disclosure statement

No potential conflict of interest was reported by the authors.

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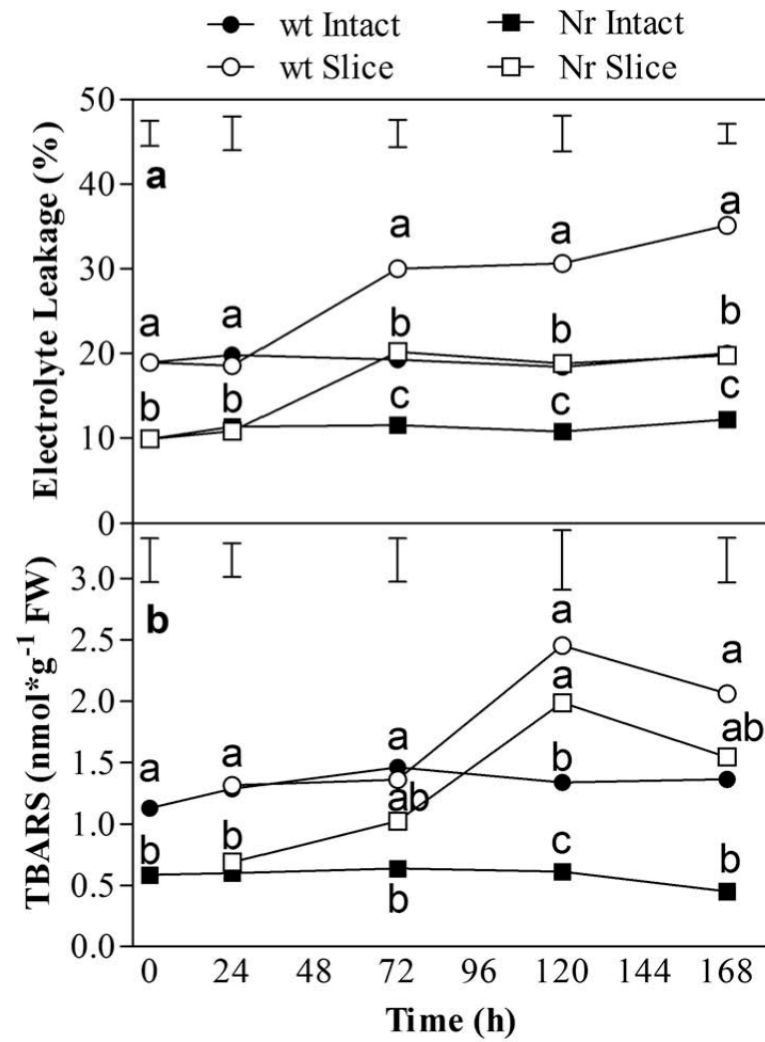


Figure 1. Effect of wounding on electrolyte leakage (a) and TBARS content (b) for intact and sliced tomato at pink stage of maturity of wt and Nr mutant. Notes: Data are mean values ($n = 4$) and vertical bars on the top of each plot are LSD values ($P \leq 0.05$) within each measuring time different letters indicate statistical differences for $P \leq 0.05$.

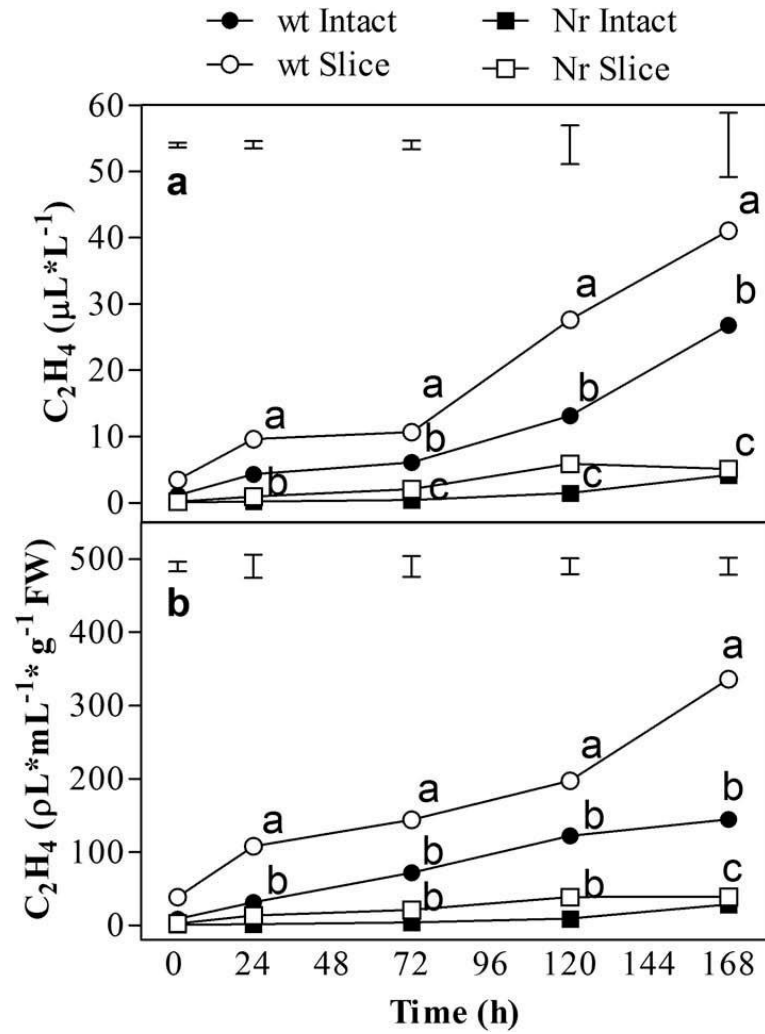


Figure 2. Effect of cutting on C_2H_4 absolute content (a) and C_2H_4 normalised content (b) in 1.1 L fresh-cut plastic containers after cutting for intact tomato and tomato slice at pink stage of maturity of wt and Nr mutant stored at 4°C in dark conditions. Notes: Data are mean values ($n = 4$) and vertical bars on the top of each plot are LSD values ($P \leq 0.05$) within each measuring time different letters indicate statistical differences for at $P \leq 0.05$.

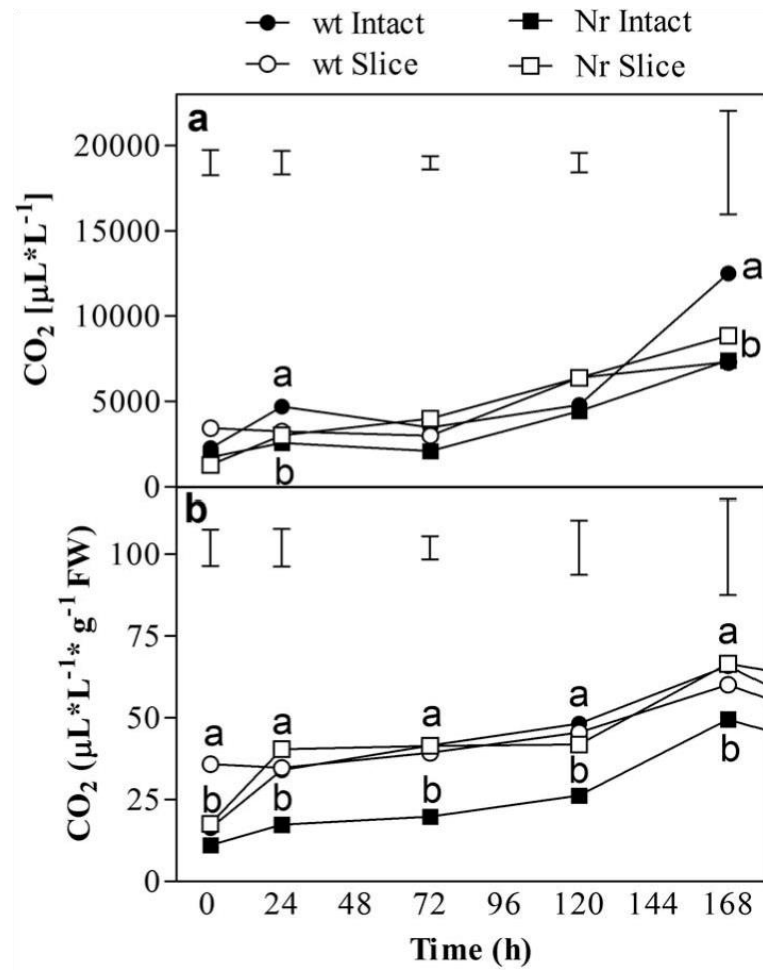


Figure 3. Effect of cutting on CO₂ absolute content (a) and CO₂ normalised content (b) in 1.1 L fresh-cut plastic containers after cutting for intact tomato and tomato slice at pink stage of maturity of wt type (nr/nr) and Nr mutant (Nr/Nr) stored at 4°C in dark conditions. Notes: Data are mean values (n = 4) and vertical bars on the top of each plot are LSD values ($P \leq 0.05$) within each measuring time, different letters indicate statistical differences for $P \leq 0.05$.

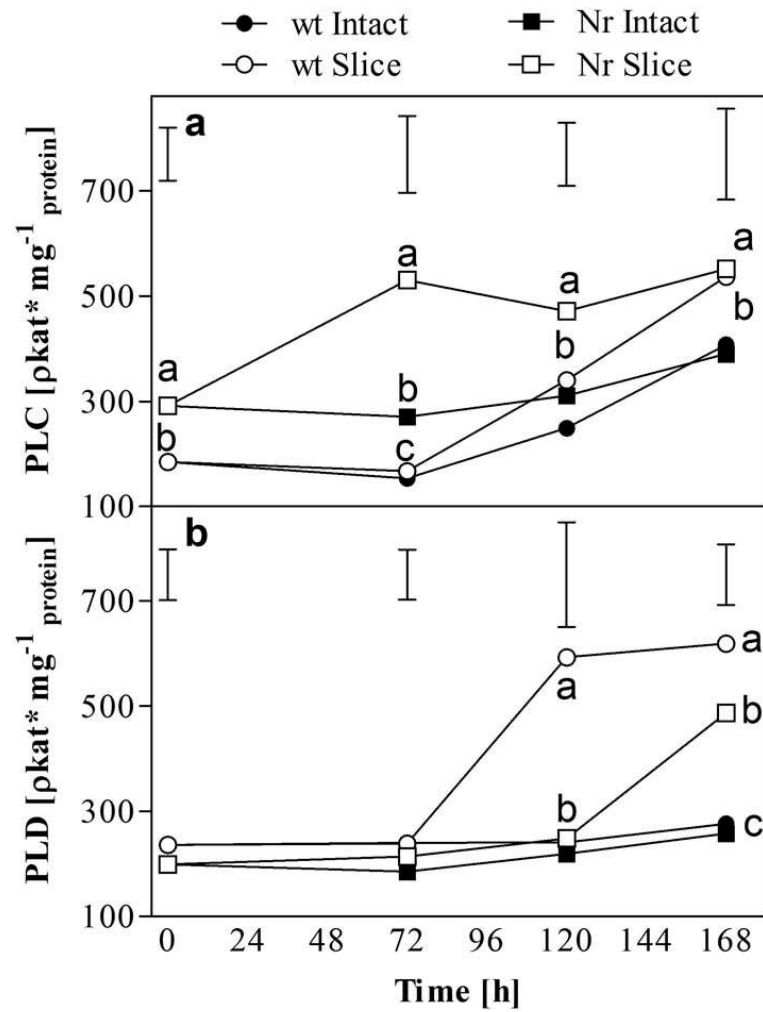


Figure 4. Effect of cutting on PLC (a) and PLD (b) enzyme activities for intact tomato and tomato slice at pink stage of maturity of wt and Nr mutant. Data are mean values ($n = 4$) and vertical bars on the top of each plot are LSD values ($P \leq 0.05$) within each measuring time different letters indicate statistical differences for $P \leq 0.05$.

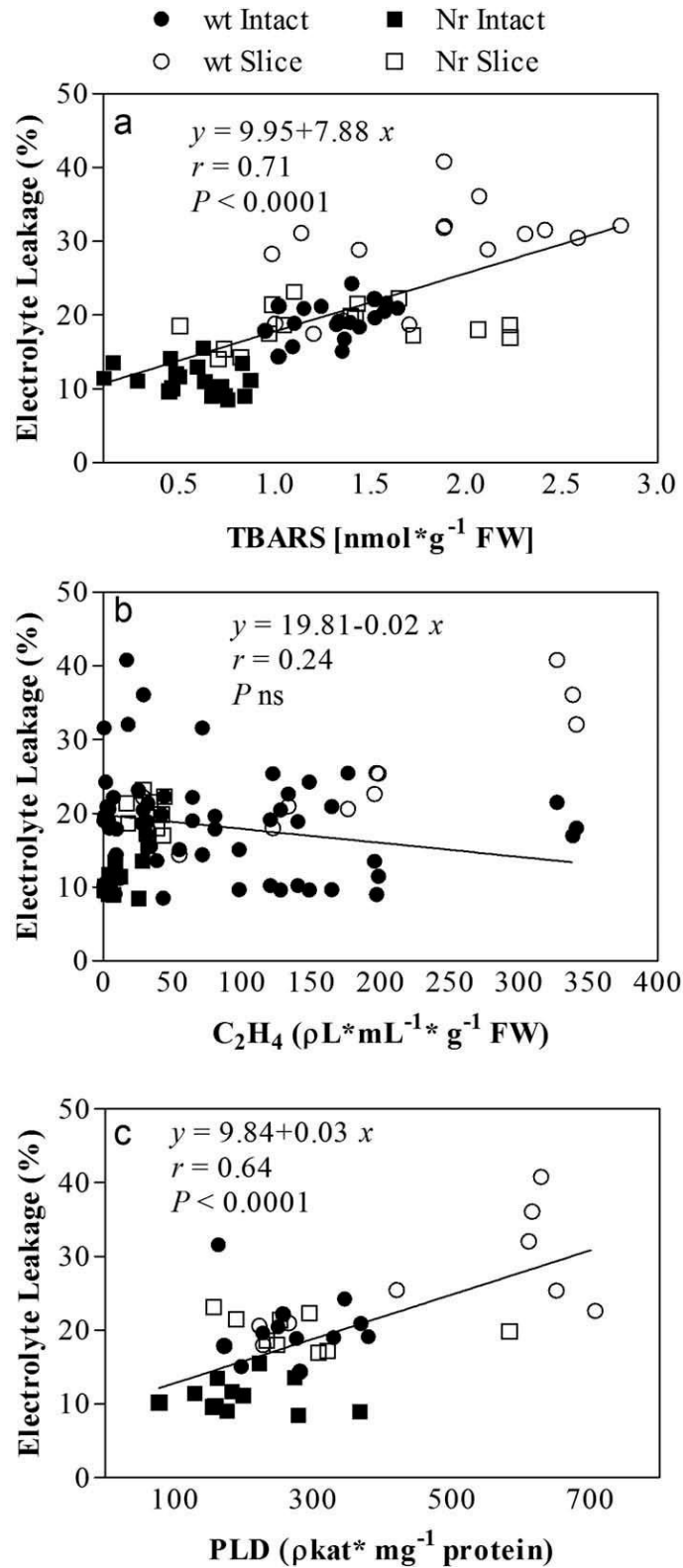


Figure 5. Correlation between electrolyte leakage and TBARS content (a) electrolyte leakage and PLD activity (b) TBARS content and PLD activity (c) for intact tomato and tomato slice at pink stage of maturity of wt and Nr mutant. Notes: Samples were stored in 1.1 L fresh-cut plastic containers at 4°C in dark conditions after cutting. Each point on the plots correspond to an individual (r, Pearson coefficient determination; P, level of significance).