CHARACTERIZATION OF THE OXIDATIVE STRESS OCCURRING AT THE ONSET OF FRUIT RIPENING: DOES IT PLAY A REGULATORY ROLE?

Stefania Pilati*[1], Daniele Brazzale[1], Adriano Sterni[2], Alberto Milli[2], Cristian Strim[2], Rita Frassanito[2], Graziano Guella[2] and Claudio Moser[1].

1 Fondazione Edmund Mach, Department of genomics and crop biology, via E. Mach 1, 38010 San Michele all'Adige (TN), Italy.
2 University of Trento, Faculty of Science, Bioorganic Chemistry Lab, via Sommarive 14, 38123 Povo (TN), Italy.
*corresponding author: stefania.pilati@iasma.it

Fleshy fruits represent an important issue of human diet, as they comprise most of the fruit we eat. According to an agronomic classification, they are divided into berries, e.g. tomatoes and grapes, drupes, e.g. cherries and peaches and the so-called “false fruits”, e.g. strawberries and apples. The development of these fruits is basically composed by two sequential phases: seed development, starting at pollination, and pulp ripening, starting at softening/coloring. During the former, embryos form and mature inside the seeds, while during the latter, pulp increase in volume, soften and accumulates sugars, flavors and pigments, which make it very attractive for animals. Seed dispersal seems to be the most likely reason for the huge investment of energy into fruit flesh made by the plant. The switch from the green to the ripening phase is under genetic control, but the mechanism/s through which such regulation is carried out are largely unknown, so far. The master switch could be represented by a signal either originated in the seeds, coming from the mother plant, or generated in the pulp itself: all these hypotheses are still open. The main reason for such a complex scenario probably resides in the fact that in a very short time many different things happen in different tissues of the fruit, so that describing them into a cause-effect relationship is extremely hard.

We previously reported the likely occurrence of an oxidative burst at veraison in grapes, deduced from ROS scavenger transcriptional modulation and H_2O_2 accumulation at veraison (Pilati et al, BMC Genomics 2007). Now we have completed this study with biochemical evidences which highlight two parallel oxidative events taking place specifically in the grape berry skin: the H_2O_2 accumulation in the cytosol and the enzymatic peroxidation of the chloroplast membranes. The transient nature of both events suggests a signalling role involved in the regulation of some pathway of the ripening process.

H_2O_2 level increases transiently in the skin of grape berry ripening at veraison (Figure 1). At the cellular level, the site of accumulation is the cytosol (Figure 2).
The activation of ROS scavengers such as catalase, superoxide dismutase and ascorbate peroxidase in the skin support the occurrence of an oxidative stress (Figure 3).

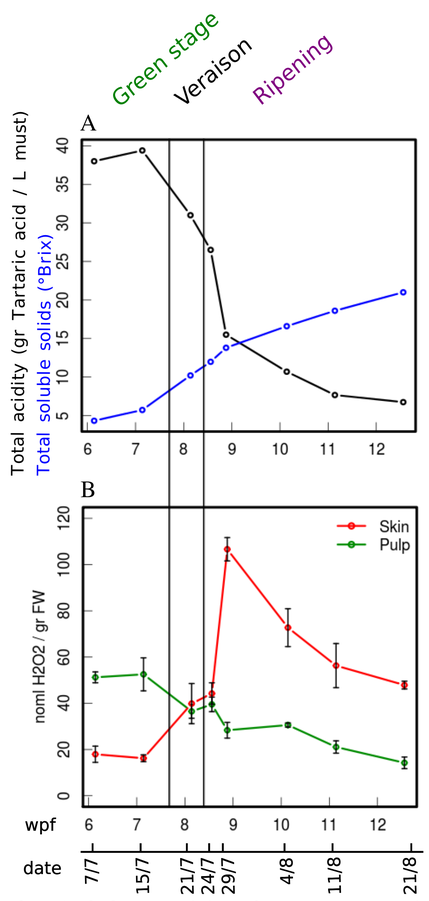


Figure 1: Biochemical changes (A) and H_2O_2 content (B) of Pinot Noir grapes during the season 2009.
A. Total acids and total soluble solids averaged on a pool of 50 berries are depicted vs. time, represented as weeks post flowering (full bloom was on May, 25th) and date of 2009 season. B. H_2O_2 was measured in the skin and deseeded pulp of PN berries using the Amplex Red method (Molecular Probes). Data are means of 3 independent H_2O_2 extractions (biological replicates) measured in technical triplicates.

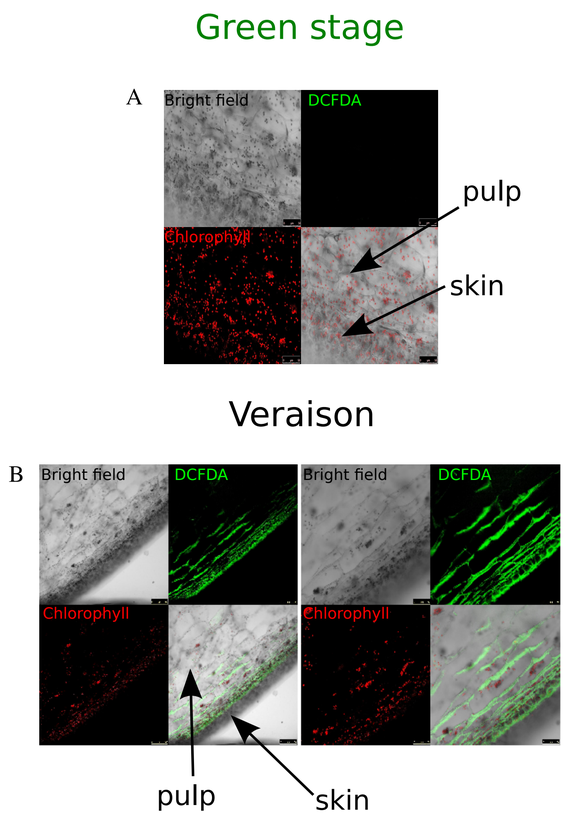


Figure 2: Confocal imaging of H_2O_2 in Pinot Noir berry sections using DCFDA, a rather specific H_2O_2 fluorescent probe. 100 microm-sections of fresh grape berries at the green (A) and veraison (B) stages were treated with DCFDA 30 microM. Images were recorded using LeicaSP II confocal microscope . Bars correspond to 50 microm.

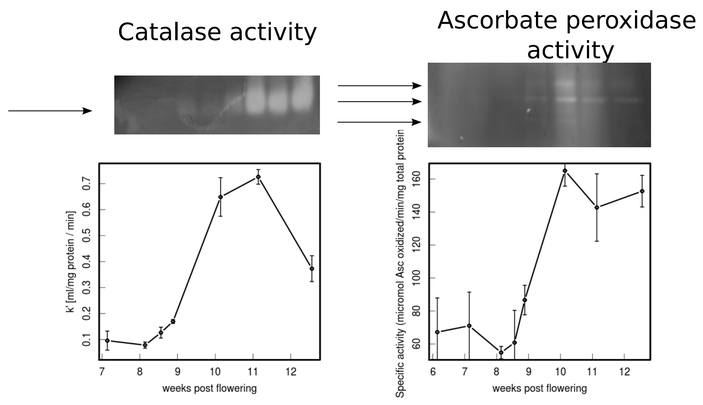
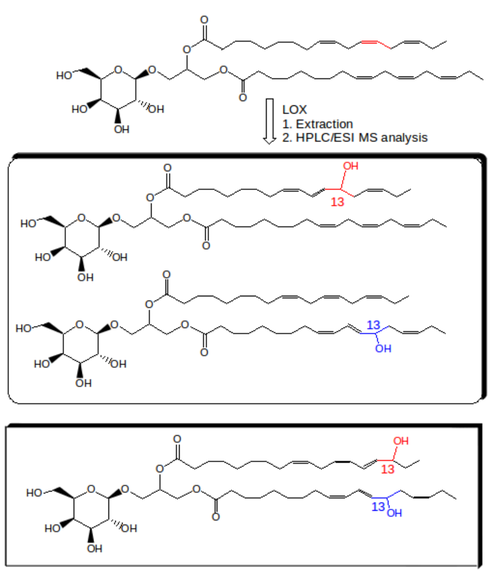


Figure 3: Grape berry skin enzymatic scavenger activities during ripening. Catalase and acorbate peroxidase activities were measured both in-gel and in vitro, while superoxide dismutase activity was measured only in-gel. For native electrophoresis 50 microgr of skin berry protein extract were loaded in each lane. In vitro activities were determined by recording absorbance at 240 and 234 nm and calculating H_2O_2 and ascorbate consumption along time, respectively.

MALDI-TOFF and HPLC/MS analyses of the lipidic fraction of grape berries along ripening revealed that only galactolipids linolenic chains were peroxidated (Figure 4).
The peroxidation is regio- and stereospecific, as only the 13(S)-HpOTRE was found (Figure 5).
Galactolipids peroxidation shows a transient increment at veraison (Figure 6), in analogy with that of H_2O_2 accumulation.
We are trying to identify a chloroplastic lipoxygenase, whose expression profile and subcellular localization propose as a good candidate to explain the lipid peroxidation (Figure 7).

Figure 4: Identification of the galactolipid fraction



MGDG 18:3/18:3, the most abundant monogalactolipid in the extract of the grape berry skin detected as $[M+Na]^+$ ions at m/z 797 in ESI (+)/MS and as $[M-H]^-$ at m/z 773in ESI (-)/MS at t_R =33 min

Mono-oxidized MGDG Ox18:3/18:3 + mono oxidized MGDG 18:3/Ox 18:3 I someric lipids detected as $[M+Na]^+$ ions at m/z 813 in ESI (+)/MS and as $[M-H]^-$ at m/z 789 in ESI (-)/MS. at at t_R =24.8 min.

Di- oxidized MGDG Ox 18:3/Ox 18:3 detected as $[M+Na]^+$ ion at m/z 829 in ESI (+)/MS and as $[M-H]^-$ at m/z 805 in ESI (-)/MS at t_R =15.3 min.

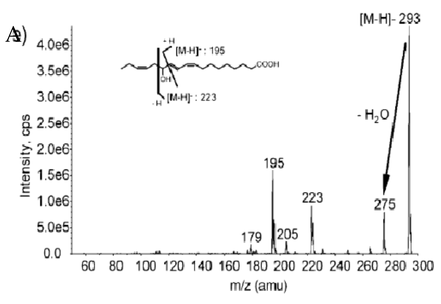


Figure 5: A:Regiochemistry of the oxidation.
Mass fragmentations occur on the carbon chain at the level of the hydroxy group providing unambiguous information about the OH-position in the fatty chain. Presence of intense ions at m/z 195 and 223 in the ESI(-) MS/MS spectrum (see Figure xx) of the oxidized 18:3 acyl chain at m/z 293 ([M-H] –) speaks for a 13-hydroxy trienoate (13- HOTE)
B: Stereochemistry of the oxidation. Chiral LC on 13-HOTE methyl ester (synthesized by using MeOH/KOH on 13-HOTE) shows that it is almost enantiomerically pure as 13-(S).Moreover, the Circular Dichroic (CD) spectrum of this compound is superimposable to that of the commercially available 13(S)-HOTE

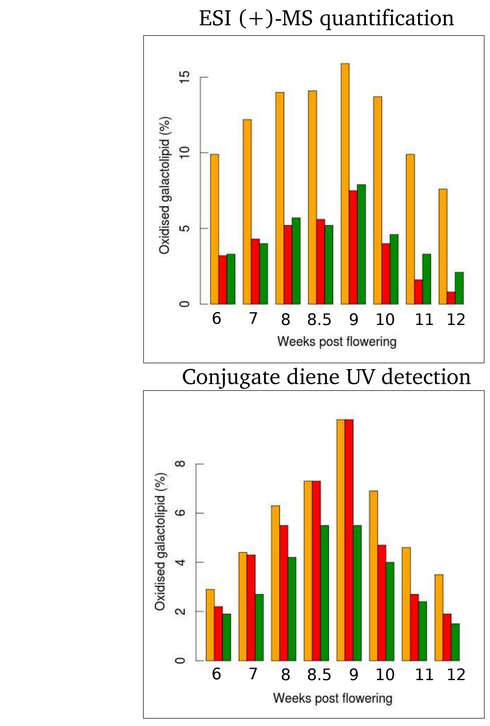


Figure 6: Lipid peroxidation profile during berry development. Quantitative measurement of the oxidized fraction was obtained by means of LC-MS (above). Formation of the conjugated diene was confirmed by UV absorption at 234nm (below).

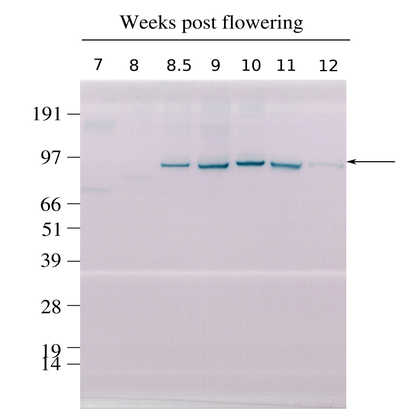


Figure 7: Western blot analysis of a 13-lipoxygenase in the grape berry skin during ripening. A commercial antibody raised against a chloroplastic 13-LOX of Arabidopsis was used to investigate LOX expression during grape berry development.

Conclusions.
We have proved the occurrence of an oxidative phenomenon at veraison, a crucial point in grape berry development at which ripening begins. However, the relationship between the two events going on in cloroplasts and cytosol is still not evident. On the one side it would be of interest to understand better the origin of such events and the consequences, if any, on gene expression/pathway regulation; on the other side it would be important to ascertain if this transient oxidative burst is common to other fruits, both climacteric and non, or if it is rather grapevine-specific.