Nitric oxide in the physiology and quality of fleshy fruits

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Highlights

- NO is a relevant regulatory molecule for fruits in chilling injury, disease resistance and ripening.
- Exogenous NO provides beneficial effects on fruit quality and seems to mimic endogenous-NO-driven physiological processes.
- NO action in fruits involves the regulation of antioxidant systems

20 **Abstract**

21 Fruits are unique to flowering plants and confer a selective advantage as it facilitates
22 seed maturation and dispersal. In fleshy fruits, development and ripening are associated
23 with numerous structural, biochemical and physiological changes, including
24 modifications in the general appearance, texture, flavor and aroma, which ultimately
25 convert immature fruits into a considerably more attractive and palatable structure for
26 seed dispersal animals. Treatment with exogenous nitric oxide (NO) delays fruit
27 ripening, prevents chilling damages, promotes disease resistance and enhances the
28 nutritional value. The ripening process is influenced by NO, which operates
29 antagonistically to ethylene, but it also interacts with other regulatory molecules such as
30 abscisic acid (ABA), auxin, jasmonic acid (JA), salicylic acid (SA), melatonin and
31 hydrogen sulfide (H₂S). NO content progressively declines during fruit ripening, with
32 concomitant increases in protein nitration and nitrosation, two post-translational
33 modifications (PTMs) promoted by reactive nitrogen species (RNS). Dissecting the
34 intimate NO interactions with other ripening-associated players, including reactive
35 oxygen species (ROS), antioxidants, and the aforementioned phytohormones, remains a
36 subject of future challenges. In this context, integrative “omics” and gene editing
37 approaches may provide additional knowledge of the impact of NO in the regulatory
38 processes involved in controlling the physiology and quality traits in both climacteric
39 and non-climacteric fruits.

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41 **Keywords:** antioxidants, chilling, disease resistance, nitric oxide, omics, post-
42 translational modifications, phytohormones, reactive nitrogen species, reactive oxygen
43 species, ripening.

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49 **Introduction**

50 Since Agriculture was set as a way of living in the Neolithic, the improvement of crops
51 has been one of the leading forces for human population growth. The search for more
52 nutritive products and new farming practices has rendered nowadays the cohabitation of
53 “latest design” and “fashion food” with the necessity of feeding unprecedentedly large
54 population nuclei which are below appropriate nurture conditions. In this framework,
55 fruits from Angiosperms have gained great relevance, and the breeding strategies
56 applied over centuries have allowed increasing productivity and nutritional value of
57 many fruit crop species, placing them as critical sources of vitamins, minerals and other
58 molecules with positive impact in human health (Karasawa and Mohan, 2018).

59 Botanically speaking, fruits are mature ovaries, which generally undergo intense
60 mitotic activity, growth and differentiation after pollination (Kourmpetli and Drea,
61 2014; Liu et al., 2015). Under physiological and evolutionary points of view, fruits are
62 structures with an extremely high diversity of shapes, colors, texture, firmness, sizes,
63 density, and chemical composition (within the same plant family, genera or even within
64 varieties of a single species), whose primary role is to maintain and spread the genetic
65 blueprint of the plant species for the next generation, even over significant distances
66 and sometimes with the help of dispersal animals (Karlova *et al.*, 2014; Kumar *et al.*,
67 2014; Giovannoni *et al.*, 2017; Corpas *et al.*, 2018; Palma *et al.*, 2018a). One of the
68 main peculiarities of most fruits is the ripening process, during which drastic
69 appearance and metabolic changes occur in coincidence with seed maturation. In
70 general terms, the ripening is a highly regulated developmental event that involves
71 reprogramming fruit biochemistry, physiology and gene expression, ultimately leading
72 to changes in coloration, nutritional composition, texture, and palatability. In fleshy
73 fruits, the ripening-associated organoleptic shifts usually rely on chlorophyll
74 degradation plus the synthesis of new pigments (*e.g.* carotenes, xanthophylls,
75 flavonoids), cell wall softening, accumulation and emission of organic volatile
76 compounds, as well as the synthesis of pectin and proteins, conversion of starch into
77 soluble sugars, together with changes in the total soluble reducing equivalents
78 (Giovannoni, 2004; Goulao and Oliveira, 2008; Barsan *et al.*, 2012; Tohge *et al.*, 2014;
79 Palma *et al.*, 2011, 2018a; Corpas and Palma, 2018; Mukherjee, 2019). Cell architecture
80 is also remodeled during fruit ripening, and in many fleshy fruits the shift in the
81 pigment composition is associated with the conversion of chloroplasts into

chromoplasts which harbor the newly synthesized carotenoids (Mateos *et al.*, 2003; Palma *et al.*, 2011, 2015; Barsan *et al.*, 2012), although other cell organelles, such as mitochondria and peroxisomes, remain structurally unchanged (Palma *et al.*, 2015). Many of these ripening-associated metabolic rearrangements affect important enzymatic and non-enzymatic antioxidant systems which are involved in maintaining the organ redox status. Those systems include superoxide dismutases (SODs), catalase (CAT), peroxidases (PODs), the enzymes of the ascorbate-glutathione cycle and a set of NADPH-generating dehydrogenases (Mateos *et al.*, 2009; Camejo *et al.*, 2010; Palma *et al.*, 2011, 2015; Huan *et al.*, 2016; Corpas and Palma, 2018; Corpas *et al.*, 2018; Muñoz-Vargas *et al.*, 2018, 2019), ascorbic acid, reduced glutathione and tocopherols (Rodríguez-Ruiz *et al.* 2018; González-Gordo *et al.* 2019; Gramegna *et al.* 2019). Although a moderate redox equilibrium is maintained during the initial phases of fruit ripening, a situation of irreversible oxidative stress takes place during the metabolic and structural dismantlement at ripe and overripe stages (Corpas and Palma, 2018; Corpas *et al.*, 2018).

Fleshy fruits are classically divided as climacteric and non-climacteric depending on their regulatory and metabolic dynamics during the initiation and progression of ripening (Alexander and Grierson 2002). This dichotomy started in the first decades of the twentieth century when a respiratory peak during fruit ripening was observed, linked to the increased emission of the plant hormone ethylene, which was later identified as a driver of the whole process. This classification based on the fruit respiration profile and ethylene dependence at ripening still prevails, grouping tomato, apple, peach, banana, mango, plum, kiwi and papaya, among others within climacteric fruits, whereas pepper, strawberry, orange, bayberry, cucumber and others, are part of the non-climacteric group (Cherian *et al.*, 2014; Liu *et al.*, 2015; Corpas and Palma, 2018). Very recently, after proving that relevant changes occurring during this physiological process were not only associated with the metabolism of reactive oxygen species (ROS) and antioxidants, but also with NO-derived mechanisms (Corpas and Palma, 2018), a nitro-oxidative stress situation was proposed to happen during both climacteric and non-climacteric ripening (Corpas *et al.*, 2018).

These latter works, together with other recent reports implicating H₂S and melatonin as part of the mechanisms of NO signaling (Ziogas *et al.*, 2018; Corpas *et al.*, 2019; Mukherjee, 2019), were mainly focused in regulation of ripening initiation and

progression and paid less attention to other ripening-associated aspects, such as chilling and disease resistance during storage and impacts on fruit organoleptic features. In this present work, an integrative updated view of the involvement of NO and other signaling players in fruit physiology during ripening and under unfavorable conditions, i.e., chilling and pathogen attacks will be given. Due to the influence of these phenomena in the fruit quality, and therefore in their exploitation as economic resources, the impact of these signaling molecules in the quality indexes will also be considered.

Role of NO in chilling injury and disease resistance of fruits

NO was reported to participate in plant responses against biotic and abiotic stress, as well as in many plant developmental events such as pollen and seed germination, deetiolation, flower setting and development, root organogenesis, nodule metabolism, cell wall lignification, senescence and fruit physiology (Manjunatha *et al.*, 2014; Kumar *et al.*, 2014; Chaki *et al.*, 2015; Melo *et al.*, 2016). Regarding fruits, the pioneer works on NO are attributed to Leshem's group who first demonstrated the involvement of this free radical in the ripening of fruits in an antagonistic way to ethylene (Leshem and Pinchasov, 2000). Since then, much effort has been devoted to investigating the participation of NO in several aspects of the fruit physiology based on data obtained from different crop species and under diverse situations, including drought, chilling, browning, disease resistance and interaction with other signaling molecules, among others (Shi *et al.*, 2011; Kumar *et al.*, 2014; Manjunatha *et al.*, 2014; Bodanapu *et al.*, 2016; Huo *et al.*, 2018; Mukherjee, 2019). Due to the quantitative and qualitative relevance of research conducted on fruits for unraveling the mechanism of action of NO and derived molecules, we will further emphasize this subject here.

Storage at low temperatures is a common practice used to delay senescence of fruits extending their postharvest quality (Chen *et al.*, 2017; Patil *et al.*, 2019), although it is often accompanied by chilling damage as a side effect. One of the most accepted effects promoted by NO in fruits is the mitigation of chilling injuries which is associated with a number of physiological impacts, thus maintaining their metabolism and quality traits for longer. Browning symptoms associated with cold damage were also alleviated by the application of exogenous NO (Peng *et al.*, 2013; Aghdam and Bodbodak, 2014; Barman *et al.*, 2014). The post-harvest treatment with NO after

storage at low temperature has been reported to reduce the chilling injury of mango peach, melon, orange and others. In **Table 1**, a summary of the most relevant effects of exogenous NO on the metabolism of ROS and antioxidants in both climacteric and non-climacteric fruits is provided. Those effects include changes in the activity pattern of SODs, CAT, ascorbate peroxidase (APX), PODs and other enzymes of diverse functions. Alongside with the enzymatic antioxidant system, the total antioxidant activity as well as the content of antioxidant compounds such as ascorbate, glutathione, phenolics, and flavonoids have also been shown to be affected by NO treatment in ripening fruits. Further practices consisting of wrapping pomegranate fruits after NO treatment also prevented those effects (Ranjbari *et al.*, 2018).

This anti-chilling role of NO seems to be leaned on diverse issues which include, among others, an enhancement of the sucrose content (Han *et al.*, 2018) and, chiefly, the antioxidative metabolism (i.e., CAT, SOD, APX, POD) (Wu *et al.*, 2012; Ren *et al.*, 2017; Zhang *et al.*, 2017a; Ghorbani *et al.*, 2018; Liang *et al.*, 2018; Venkatachalam, 2018), although some report found that sodium nitroprusside (SNP), used as a common NO source, behaved in an opposite way with the antioxidant enzymes being depressed (Saba *et al.*, 2017; Tareen *et al.*, 2017) (**Table 1**). It has been also reported that in the signaling pathway which activates chilling tolerance in cucumber (*Cucumis sativus*), jasmonic acid (JA) functions upstream of and depend on NO, and this reduces H₂O₂ levels due to an enhanced catalase activity and gene expression (Liu *et al.*, 2016). The pre-harvest combined fumigation of peach fruits with NO and the ethylene-synthesis inhibitor aminoethoxyvinylglycine (AVG) generated a higher radical scavenging activity during cold storage and a similar pattern was also observed with the simultaneous application of AVG and salicylic acid (SA), but no consistent trends of the antioxidant enzymes were recorded in those studies (Tareen *et al.*, 2017).

On the other hand, stimulation of nitrogenous compounds such as polyamines, proline and γ -aminobutyric acid (GABA) has been proved to take place in bamboo and banana fruits subjected to chilling conditions as a consequence of NO pretreatment (Wang *et al.*, 2016; Wang *et al.*, 2017). Additionally, the use of NO and GABA in Cornelian cherry fruits was accompanied by a delayed senescence during post-harvest cold storage concomitant with an enhanced activity of the antioxidant enzymes SOD, CAT, APX and glutathione reductase (GR) (Rabiei *et al.*, 2019). Also H₂S, which is involved in the regulation of growth and development in plants, has been lately found to

180 enhance chilling tolerance by promoting the activity of antioxidant enzymes and
181 increasing the proline and polyphenols contents (Huo *et al.*, 2018; Corpas *et al.*, 2019).
182 Aghdam and Bodbodak (2014) proposed that, among other parameters, the mitigation of
183 chilling in heat-treated fruits was due to the augmentation of the arginine pathways
184 which gives rise to the accumulation of signal molecules like NO, polyamines and
185 proline. In a further study, it has been reported that the use of exogenous melatonin
186 confers chilling tolerance to tomato fruits by promoting polyamine and proline levels as
187 well as the accumulation of NO by triggering the arginine pathway activity (Aghdam *et*
188 *al.*, 2019).

189 The beneficial effect of NO in fruit disease resistance against a number of
190 pathogens, basically fungi, has been also demonstrated in diverse species including
191 tomato, pitaya, mango, citrus, peach, kiwi, and others. In **Table 2**, the impact of NO
192 treatment on plant disease resistance is briefly reviewed as it has been associated to the
193 induction of a series of defense-related genes/enzymes (Zheng *et al.*, 2011a, 2017; Yu *et*
194 *al.*, 2012; Gu *et al.*, 2014; Hu *et al.*, 2014, 2019; Li *et al.*, 2017). Besides this effect on
195 the defense system, alterations of ROS and antioxidant metabolisms have been reported
196 as a consequence of the exogenous NO application in fruits (Fan *et al.*, 2008; Lai *et al.*,
197 2011; Zhang *et al.*, 2011, 2017b; Shi *et al.*, 2015; Zhou *et al.*, 2016) (**Table 2**). The
198 impact of NO in fruit disease resistance resembles the one reported in whole plants
199 (Romero-Puertas and Delledonne, 2016; Mata-Pérez *et al.*, 2016). The cooperative
200 interaction of NO with other signaling molecules and phytohormones (H₂O₂, JA, SA)
201 have been found to induce disease resistance in diverse fruit species (Fan *et al.*, 2008;
202 Shi *et al.*, 2015; Zheng *et al.*, 2014, 2015).

203 More research is necessary to fully understand how those and other potential
204 interactions take place, and how they are influenced depending on the crop species and
205 their secondary metabolites which may modulate the specific role of those regulatory
206 molecules, thus contributing to the maintenance of the fruit quality.

207 The search for new varieties resistant to pest through classical breeding
208 technologies has been a significant focus of attention for plant growers and
209 biotechnological companies. Although still fragmented, the emerging role of NO as an
210 important player in plant response against pathogens can help to identify potential gene
211 targets for transgenic manipulation (e.g., overexpression, silencing via VIGS or RNAi)

of gene editing approaches (e.g., CRISPR/Cas9). This may allow gaining knowledge for further breeding purposes with quality traits as final objectives and could also help minimizing economical losses due to precocious fruit deterioration during production and transportation. Thus, for example, overexpression of the mitogen-activated protein kinases (MAPKs) could improve the NO-induced disease resistance. In fact, the involvement of MAPKs in the NO-dependent response of tomato fruits against *Botrytis cinerea* was reported (Zheng *et al.*, 2014, 2015).

NO and fruit ripening. Interaction with phytohormones and regulatory molecules

As indicated above, early works carried out at the Professor Ya'acov Y. Leshem's laboratory in the Bar-Ilan University opened up a window of opportunity for controlling fruit ripening by demonstrating that NO and ethylene acted antagonistically during this physiological process (Leshem and Pinchasov, 2000). This group reported that exogenously applied NO, either as sodium nitroprusside (SNP), NONO-ate or nitrosoglutathione (GSNO), delayed flower and leaf senescence as well as fruit ripening (Leshem, 2000; Leshem and Pinchasov, 2000). Interestingly, it was found that in kiwifruit ripening was considerably inhibited by O₃, and this was reversed by SNP (Tanou *et al.*, 2015). The antagonism between NO and ethylene in climacteric fruits was later corroborated (Prochazkova and Wilhelmova, 2011), and the influence of NO in specific steps of the ethylene biosynthetic pathway has been described (Lindermayr *et al.*, 2006; Cheng *et al.*, 2009). Illustrative examples where NO down-regulates fruit ethylene synthesis include apple (Rudell and Mattheis, 2006), peach (Zhu *et al.*, 2006), strawberry (Zhu and Zhou, 2007), tomato (Eum *et al.*, 2009), among others (Mukherjee, 2019 and references therein).

The ethylene biosynthesis involves two steps (**Fig. 1**). In the first one, the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) converts S-adenosyl-methionine (SAM) into ACC; whereas in the second step, ACC is oxidized to ethylene by the ACC oxidase (ACO) (Lindermayr *et al.*, 2006; Arc *et al.*, 2013). SAM is continuously replaced through a methionine cycle (Yang cycle) where SAM synthetase (SAMS) adenylates the methionine to render the first precursor of the ethylene synthesis (Freschi, 2013; Rodrigues *et al.*, 2014; Arc *et al.*, 2013). As illustrated in **Fig. 1**, NO seems to modulate several points of ethylene metabolism, including the reversible

244 inactivation of SAMS, also known as methionine adenosyltransferase (MAT), via
245 reversible S-nitrosation modification (Lindermayr *et al.*, 2006). Among the three MAT
246 isoforms (MAT1-MAT3) responsible for SAM biosynthesis in Arabidopsis, Lindermayr
247 and colleagues (2006) demonstrated that the S-nitrosation of only MAT1 at the Cys-114
248 position provoked activity inhibition. On the other hand, NO-induced inhibition of both
249 ACS and ACO activity and gene expression has also been described during the ripening
250 of several fleshy fruits (Cheng *et al.*, 2009; Freschi, 2013; Mukherjee, 2019).

251 Other regulatory partners may participate in the NO-ethylene dialog during fruit
252 ripening. For example, the stress hormone abscisic acid (ABA) has been increasingly
253 demonstrated to maintain crosstalk with both NO and ethylene, at least during
254 vegetative plant responses such as seed dormancy and germination (Arc *et al.*, 2013;
255 Prakash *et al.*, 2019). Likewise, the application of nitric oxide seems to counteract the
256 ABA-induced senescence in rice (*Oryza sativa*) leaves (Hung and Kao, 2003), whereas
257 in barley (*Hordeum vulgare*) NO can retard programmed cell death of aleurone layers
258 (Beligni *et al.*, 2002). Nevertheless, little is known about this interplay among NO-
259 ethylene-ABA in fruits. Moreover, ethylene and auxin have also been shown to closely
260 interact to modulate ripening-associated processes, including carotenoid biosynthesis in
261 tomato (Su *et al.*, 2015; Cruz *et al.*, 2018). As the auxin-NO signaling interplay also
262 regulates many aspects of plant vegetative development (Freschi *et al.*, 2013), we must
263 remain open-minded to conceive even more complex NO-hormone regulatory networks
264 controlling ripening-related processes in fleshy fruits. Also, the combined pre-treatment
265 of mango fruits with NO and SA modulated the expression of up to six genes involved
266 in the ethylene biosynthesis and the authors proposed that those genes could participate
267 in the NO and SA signaling pathways during ripening (Hong *et al.*, 2014). Additional
268 potential interactions between ethylene metabolism and plant regulatory molecules,
269 including NO, ABA, auxin, JA, SA, gibberellic acid, cytokinins, brassinosteroids, and
270 polyamines during fleshy fruit development and ripening can be found elsewhere
271 (Kumar *et al.*, 2014).

272 More recently, it has been proposed that the crosstalk between NO and ethylene
273 during the ripening process is mediated by H₂S and melatonin (Huo *et al.*, 2018;
274 Mukherjee, 2019; Liu *et al.*, 2019). In this interaction, NO participates through its action
275 over the ethylene biosynthesis, but it also promotes post-translational modifications of
276 antioxidant enzymes in junction with H₂S. The NO-mediated PTMs of these enzymes

may in turn lead to changes in cell wall firmness, pericarp browning, anthocyanin, and flavonoid biosynthesis as well as in the profiles of ROS/RNS, lipid peroxidation and chlorophyll degradation, aspects which are associated to the delayed ripening promoted by those two regulatory molecules, NO and H₂S. In this scenario, melatonin seems to interact with NO influencing the activity of diverse antioxidant enzymes, ultimately leading to similar symptoms to those displayed by NO and H₂S (Mukherjee, 2019). In fact, melatonin has been proved to delay postharvest senescence of pear fruits by triggering the NO-mediated inhibition of the ethylene synthesis genes. Consequently, melatonin seems to act upstream of NO in plant systems (Liu *et al.*, 2019). Moreover, during sweet pepper fruit ripening, H₂S production capacity has been reported to increase as a consequence of a higher L-cysteine desulfhydrase activity. In this system, H₂S inhibits the activity of the NADPH-generating isocitrate dehydrogenase (NADP-ICDH) possibly by a persulfhydration process, what indicates that H₂S may regulate the NADPH production during fruit ripening in a coordinated way with NO (Muñoz-Vargas *et al.*, 2018, 2019).

Given the relevant and very well-established role of ethylene on climacteric ripening, fruit crops belonging to this group, including the genetic model tomato, may represent more promising systems to dissect the mechanisms behind the NO-ethylene interaction than non-climacteric fruits. But, comparative studies on the NO action on both climacteric and non-climacteric fruits should also occupy a prominent place in future years of NO and fruit biology research.

Accumulating evidence indicates that, regardless of belonging to the climacteric or non-climacteric groups, fleshy fruits display some common physiological and metabolic responses to exogenous NO treatment. By comparing the effects triggered by the NO treatment on several crop fruit species (**Table 1**), it becomes clear that NO frequently prolongs fruit post-harvest lifespan and sometimes provides added nutritional value to them, and both these effects have obvious commercial and human health implications (Manjunatha *et al.*, 2014; Corpas and Palma, 2018; Corpas *et al.*, 2018; Steelheart *et al.*, 2019). Additionally, as indicated above, the treatment of diverse fruits with NO triggers other symptoms of economic/commercial relevance such as preventing browning in apple, lowering chilling injury in banana, increasing firmness in blueberry and bayberry, softening delay in papaya, mango and strawberry, and inducing disease resistance during postharvest storage, among others (**Table 1**). Accordingly,

310 treatment with NO promotes, at least, the maintenance of organoleptic properties and
311 their nutritional value.

312 Very recently, transcriptomic and proteomic approaches have started to reveal
313 how NO may affect the intimate metabolism of sweet pepper during ripening. The
314 analysis of differentially expressed genes (DEGs) by RNA-Seq showed that about 2,200
315 genes were up-regulated and around 3,300 were down-regulated in the transition from
316 immature green fruits to ripe red ones (Corpas *et al.*, 2018). However, the treatment of
317 fruits with 5 ppm NO lowered the number of down-regulated genes (above 1,600), but
318 2,125 were up-regulated (González-Gordo *et al.*, 2019). This indicates that NO, either
319 directly or indirectly, leads to extensive changes in gene expression during fruit
320 ripening. Moreover, proteomic analysis by iTRAQ rendered that, from the 2,574
321 identified proteins in pepper fruits, about 692 and 497 were differentially
322 overrepresented in green and red ripe fruits, respectively. After NO treatment, only 24
323 proteins were more abundant in green pepper while 55 displayed lower levels (Palma *et al.*, 2018b). Although transcriptomic and proteomic data are always difficult to match
324 and to infer from one to another, it seems plausible to assume that besides influencing
325 the global transcript abundance NO may also play a significant role on the translational
326 control of protein accumulation in ripening fruits. However, more research is necessary
327 to validate this hypothesis.
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330 **Endogenous vs. exogenously added NO**

331 Several aspects need to be taken into consideration when comparing data from the
332 experiments based on NO treatments referred above: (a) those experiments were carried
333 out with exogenously-added NO levels which usually overpass those operating under
334 physiological conditions; (b) the route followed by the NO molecules after its
335 application is difficult to be experimentally investigated, and, at present, the fraction of
336 the applied NO that actually gain access to the target cells inside large fruits is far from
337 being determined; (c) the average halflife of NO in aqueous solution is about 10
338 seconds, but this value can be totally different within fruit cells, where osmolytes,
339 sugars, diverse antioxidants and compounds (secondary metabolites) specific for each
340 fruit species can interfere with the NO stability and action; (d) the effects observed after
341 NO treatment are usually detectable after some days, so some still uncharacterized

on/off switch seems to be activated by the gas; (e) once inside the cell, NO can either give rise to a series of RNS promoting PTMs of some proteins (S-nitrosation, nitration or nitro-alkylation) and lipid nitration or interact with ROS. So that, in the current state of the art, as depicted in **Figs. 2A, 2B**, two plausible ways of investigating the role of NO in fruit ripening can be highlighted: 1) by treatment with exogenous NO, as it has been thoroughly reported here, and considering the cues (a) to (e) referred above; and 2) by promoting the production of endogenous NO. Regarding this latter issue, Bonadapu *et al* (2016) found that the *short root* (*shr*) tomato mutant displayed overproduction of NO and this gave rise to suppression of fruit growth and ripening. Alternatively, the level of endogenous NO can be enhanced by several pre-harvest treatments. Thus, treatment with L-arginine alone and combined with methyl salicylate was positively correlated with endogenous NO concentration in tomato, and this promoted the fruit resistance to *Botrytis cinerea* (Zheng *et al.*, 2011b; Zhang *et al.*, 2017). Furthermore, the treatment of tomato fruits with the elicitor from *B. cinerea* led to a rapid biosynthesis of NO and also activated the defense enzyme system (Zheng *et al.*, 2011a). Likewise, the application of the yeast saccharide from *Penicillium expansum* triggered a marked increase of endogenous NO in peach fruits, and this reduced decay during postharvest storage (Yu *et al.*, 2012). In apple, it was reported that the metabolism of endogenous NO during growth and development of fruits influenced the internal profiles of nitrosothiols (SNOs), L-arginine, and nitrite and nitrate contents (Zhang *et al.*, 2011).

From the metabolic and physiological points of view, the beneficial effects of NO, either from exogenous or endogenous sources, during the ripening process are also related with the ROS metabolism and antioxidants, thus weaving a multifaceted chain of reactions which remains to be better characterized in the context of fruit biology. For example, it has been demonstrated that treatment with 5 ppm NO provokes an increase of about 40% in the ascorbate content of sweet pepper fruits (Rodríguez-Ruiz *et al.*, 2017a), thereby incrementing the fruit nutritional content. Additional reports on the interactions among RNS and ROS in fruits will be addressed below.

Fruit ripening, RNS and ROS

Like most other plant organs, fruits are able to generate NO, although there is still no unequivocal data on the specific sources, either enzymatic or non-enzymatic, as it still

prevails in higher plants where diverse sources have been proposed (Astier *et al.*, 2018; Steelheart *et al.*, 2019). However, what has been demonstrated via different detection approaches is that NO level decreases during fruit ripening (Chaki *et al.*, 2015; Muñoz-Vargas *et al.*, 2018). Concomitantly, increased amounts of both protein nitration and SNOs are detected as fruits ripen (Chaki *et al.*, 2015; Rodríguez-Ruiz *et al.*, 2017b; Corpas and Palma, 2018; Corpas *et al.*, 2018). Protein nitration is considered thus far an irreversible PTM in plants which occurs by the covalent addition of a nitro (-NO₂) group to specific tyrosines in the polypeptide chain, and it is mediated by the formation of peroxynitrite (ONOO⁻) as a consequence of the reaction between superoxide radicals (O₂⁻) and NO (Lozano-Juste *et al.*, 2011; Corpas *et al.*, 2013; Chaki *et al.*, 2015). On the other hand, protein S-nitrosation is a reversible PTM involving the covalent addition of a NO group to the thiol (-SH) side chain of cysteine present in peptides or proteins giving rise to a family of NO-derived molecules called protein SNOs (Astier *et al.*, 2011). Both PTMs can modify protein functions, either positively favoring their activity or negatively by interacting with specific aminoacids and turning them missfunctional.

According to the respective profiles of NO, protein nitration, and S-nitrosation, it could be hypothesized that as ripening progresses the NO present at a high level in unripe fruits is driven to promote both PTMs, so NO is consumed in the process, thereby explaining the lower concentrations found in ripe fruits (**Fig. 2C**). Nitration of fatty acids has also been reported to occur in plant tissues and, in fact, the presence of nitro-fatty acids (NO₂-FAs) has been proved in extra virgin olive oil (EVOO) (Mata-Pérez *et al.*, 2017). NO₂-FA can bind, via a Michael addition, to a thiol group of proteins through nitro-alkylation which could be part of a signaling process (Sánchez-Calvo *et al.*, 2013; Mata-Pérez *et al.*, 2017) such as it has been reported in animal cells (Geisler and Rudolph, 2012). Although this nitro-alkylation event has not been investigated during the ripening of olive fruits, it seems tempting to speculate that such reaction may play a role in fine-tuning the regulatory mechanisms behind the drastic metabolic changes triggered by ripening in fleshy fruits. Whether or not the NO applied to fruits follows the pattern shown in **Fig. 2C** remains to be investigated, and this would allow addressing most of the cues (a) to (e) indicated above. For example, when sweet pepper was exposed to exogenous NO, the ripening process was delayed several days, and the pattern of nitrated proteins was influenced by such treatment. The profile of nitrated proteins in pepper fruits treated with 5 ppm NO, which is an extremely high

concentration comparing to internal NO levels, resembled that of immature green fruits rather than that of ripe red fruits (Chaki *et al.*, 2015). Initially, these findings may seem somehow contradictory, as the higher NO availability would imply a higher formation of ONOO⁻ which could favor protein nitration. This might be true if the generation rate of superoxide radicals is also enhanced since the reaction between NO and O₂⁻ is equimolar, so the level of O₂⁻ is possibly limiting the ONOO⁻ production.

Interestingly, five-fold increased activity of the Rboh has been reported in sweet pepper after fruits ripened (Chu-Puga *et al.*, 2019). Rboh, corresponds to a respiratory burst oxidative homologue, a protein found in plants that shares functional similarity to the animal NADPH oxidase, and is involved in the generation of O₂⁻ (Suzuki *et al.*, 2011; Zhang *et al.*, 2018). However, once again, the O₂⁻ concentration at which this species is generated intracellularly might never be in the same range as the 5 ppm NO applied to fruits. So, this behaviour observed in the profile of protein nitration leads us to think that only a little part of the NO molecules administered to the fruits gained access to the target cells inside this organ where they were integrated in the RNS metabolism and operated through diverse mechanisms, including nitration and S-nitrosation of proteins, and perhaps also by nitro-alkylation of fatty acids. The combination of NO with reduced glutathione (GSH) to form GSNO, and the subsequent removal of this molecule via GSNO reductase (GSNOR) activity is also a possibility that deserves further investigation. However, GSNOR activity has been shown to decline during pepper fruit ripening (Rodríguez-Ruiz *et al.*, 2017b), thereby potentially limiting the tissue capacity to reduce the cellular levels of this NO reservoir as ripening progresses.

Considering that protein nitration in plants has been associated with biotic and abiotic stress, and that both unfavorable conditions develop oxidative stress symptoms, this PTM has been used traditionally as an oxidative stress marker (Corpas *et al.*, 2009, 2013; Chaki *et al.*, 2015). Moreover, enhanced lipid peroxidation takes place during fruit ripening (Corpas *et al.*, 2018; Chu-Puga *et al.*, 2019), thus indicating that a moderate situation of oxidative stress is linked to fruit ripening. This combination of increased oxidative and RNS metabolisms allowed concluding that nitro-oxidative stress undergoes at ripening. However, due to the physiological meaning of the ripening for the survival of plant species throughout generations, the term “*phystress*” is

439 proposed in this review to refer to the sort of *physiological stress* that takes place in
440 ripening fruits.

441 Under natural conditions, it has been found that catalase, an enzyme that
442 catalyzes the disproportionation of H_2O_2 into H_2O and O_2 , is the major protein nitrated
443 during the ripening of pepper fruits (Chaki *et al.*, 2015). This PTM is responsible for the
444 lower catalase enzyme activity determined in ripe fruits (Mateos *et al.*, 2003; Chaki *et al.*,
445 2015). Another protein involved in controlling cellular H_2O_2 levels is the
446 chloroplastic peroxiredoxin II E (Prx-IIIE). In preliminary studies, also developed in
447 sweet pepper, it was reported that Prx-IIIE was potentially *S*-nitrosated during the
448 ripening process, although thus far it is not precisely known if this modification results
449 either in gain or loss of function (Rodríguez-Ruiz *et al.*, 2017b). This connection
450 between catalase and Prx-IIIE is an interesting point since NO and H_2O_2 are among the
451 most representative species from the RNS and ROS families, respectively, and are
452 increasingly implicated in cell signaling events (Camejo *et al.*, 2010; Airaki *et al.*, 2015;
453 Huan *et al.*, 2016; Jiménez-Quesada *et al.*, 2017; Corpas *et al.*, 2018; Palma *et al.*, 2019;
454 Steelheart *et al.*, 2019).

455 The cause (NO) and consequence (ROS and antioxidants) relationship has been
456 more widely investigated in cases where NO has been exogenously added in different
457 forms (**Table 1**). One of the most remarkable examples of this relationship was reported
458 in the non-climacteric sweet pepper. In this fruit, the treatment with NO allowed
459 increasing up to 40% in ascorbate content, which was associated with higher gene
460 expression and enzyme activity levels of the galactono- γ -lactone dehydrogenase, the
461 enzyme responsible for catalyzing the last step of the ascorbate biosynthesis in plants
462 through the L-galactose pathway (Rodríguez-Ruiz *et al.*, 2017a). However, the effect of
463 NO treatment in the ROS and antioxidant metabolism may be extended to other
464 parameters, although it must be said that the available information on the intimate
465 mechanisms behind such relationships is still unknown. In tomato, for instance,
466 although a number of papers establishes a clear interaction among NO, ROS and other
467 signaling molecules (primarily hormones) in different vegetative tissues (Gémes *et al.*,
468 2011; Poór *et al.*, 2015; Piterkova *et al.*, 2013; Corpas *et al.*, 2018), such signaling
469 interplays have not been determined in the fruit tissues yet. Again, the use of new
470 “omic” and gene editing technologies can be very useful to understand those processes
471 as the combination of genetic engineering of NO, ROS and hormone metabolisms

followed by detailed screening of the impacts of such manipulations on fruit transcriptome, proteome and metabolomic profiles may provide particularly comprehensive information about NO-ROS-hormone interplay during the fruit development and ripening.

Appraisal and future perspectives

Independently on whether fruits are climacteric or non-climacteric, NO has been demonstrated to delay ripening and to affect their redox dynamics by improving their antioxidant activity and, therefore, favoring their nutritional value. The limited data currently available on RNS and ROS metabolisms of pepper fruits under natural conditions (Chaki et al., 2015; Mateos et al., 2009; Rodríguez-Ruiz et al., 2017b; Corpas and Palma, 2018) indicate that the NO added exogenously to fruits develops the same symptoms that perhaps occur physiologically during ripening. Therefore, it is plausible that the approach of exposing fruits to unusual concentrations of NO, commonly above physiological levels, may be useful to mimic the internal conditions of this organ. Nevertheless, this has to be investigated in more detail given the diversity of agronomically-relevant fruits and their significant differences in molecular composition. Thus, a considerable variation in the content of sugars, organic acids, antioxidants, lipids, vitamins, polyphenols, fibers, alkaloids and others might determine the final response of fruits to the NO treatment. Conducting the approaches reported above on the stimulation of the endogenous NO sources by treatment with eliciting molecules on fruit ripening mutants, or, for example, silencing genes which may provoke imbalances in the ROS/RNS metabolism (*SOD*, *CAT*, *GSNOR*), can also provide irrefutable data which allows depicting the scenario at each development and ripening stages.

The interaction between NO and the endogenous antioxidants, as revealed in this review, portrays an exciting perspective, since it allows drawing, together with the ROS metabolism, a theoretical and balanced picture which could be disturbed under stress conditions, and also by the presence of other key molecules such as phytohormones, H₂S and melatonin, as it has been reported very recently (Muñoz-Vargas *et al.*, 2018; Mukherjee, 2019; Liu et al., 2019). In pepper fruits, which are one of the richest plant sources of ascorbate, the interaction between this soluble antioxidant and the exogenously added NO, and other RNS-derived PTMs such as *S*-nitrosation and

504 nitration, offers an exciting target for future investigations. In fact, ascorbate has been
505 proposed to function as a redox buffer which modulates the fruit ripening process in
506 pepper (Palma *et al.*, 2015).

507 On the other hand, the focus of the research on the NO impact on fruit
508 physiology can be significantly diversified depending on the final objectives. From a
509 scientific and academic point of view, the strategy of applying NO to fruits, as
510 performed so far by different groups is, to our concern, adequate since it enables us to
511 gain knowledge on this regulatory facet of the ripening process, and it can also provide
512 virtual scenarios which could be near the *in vivo* situation. Nevertheless, if the interest
513 comes from agronomical and economic viewpoints, where a significant goal may be to
514 facilitate postharvest storage and improve fruit nutritional values via NO treatment, it
515 must be taken into consideration that subjecting tons of fruits with gaseous NO is not a
516 feasible practice. Then, new alternatives need to be developed, including either the
517 discovery of natural NO sources which can be cheap and easily handled or the use of
518 NO production elicitors.

519 Clarifying how these NO-triggered events mechanistically influence fruit
520 physiology and metabolism will inexorably require the use of a wide variety of
521 methodological approaches and strategies, some of which are currently available and
522 others yet to be developed. Modern approaches based on “omics” tools have the
523 potential to help to elucidate many of the open questions in this subject. Very recently, a
524 study of the effect of NO in the transcriptome of sweet pepper fruits has been
525 accomplished, and a set of genes displaying differential expression due to the NO
526 treatment has been reported. Some of them are related to chloroplast metabolism, lipid
527 metabolism, cysteine metabolism, response to H₂O₂, and regulation of NO metabolic
528 processes (González-Gordo *et al.*, 2019). Integrating transcriptomics, proteomics and
529 metabolomics in this field will, perhaps, be part of the solution, and, of course, using the
530 large datasets generated via these approaches to identify the mechanisms behind NO
531 action in fruits represents an imminent challenge for both NO and fruit biologists.

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542 **Figure legends**

543 **Figure 1.** Ethylene biosynthesis pathway and methionine recycling in plants. Ethylene
544 is synthesized in a two-step pathway which involves the enzymes ACC synthase and
545 ACC oxidase. The S-Adenosyl methionine (SAM) is continuously replaced by a
546 methionine cycle (Yang cycle), although SAM can also be derived to the biosynthesis
547 of the polyamines spermidine and spermine. Nitric oxide (NO) inhibits the ACC
548 synthase and ACC oxidase activity, and it also affects the SAM synthetase, also known
549 as methionine adenosyltransferases (MAT), by a S-nitrosation event. On the other hand,
550 melatonin can stimulate the biosynthesis of NO. ACC, 1-aminocyclopropane-1-
551 carboxylic acid; MTA, 5-methylthioadenosine; MTR, 5-methylthioribose; KMB, 2-
552 keto-4-methylthiobutyrate.

553 **Figure 2.** Metabolism of NO in fruits. The levels of internal NO can be modified by
554 either (A) subjecting fruits to high exogenous NO levels, or (B) stimulating the
555 endogenous NO production by the use of L-arginine, methyl salicylate (MeSa), the
556 elicitor of *Botrytis cinerea* (B.c.) or yeast saccharide (YS). (C) NO, which is in a higher
557 concentration in immature green fruits than in red ripe, can be driven to protein Tyr-
558 nitration and S-nitrosation processes which are increased at ripening. Besides, nitro-
559 alkylation of proteins mediated by fatty acids (FA) may occur as reported elsewhere. In
560 either green or red fruits, NO can also participate in signaling trials.

Table 1. Effects of NO treatment on physiological traits and interactions between NO and the oxidative metabolism in some climacteric and non-climacteric fruits

Fruit	NO donor	Effects	Main interactions with oxidative metabolism (ROS and antioxidants)	Reference
Climacteric				
Apple (<i>Malus domestica</i>)	DETANO	- Inhibition of browning in apple slices - Increases postharvest life	-	Pristijono <i>et al.</i> , 2008
Banana (<i>Musa spp.</i>)	SNP	- Enhances chilling tolerance - Lowers ethylene production and malondialdehyde content	- Lowers O ₂ ⁻ production and hydrogen peroxide (H ₂ O ₂) content - Increases activities of SOD, CAT, APX and POD	Cheng <i>et al.</i> , 2009 Wu <i>et al.</i> , 2014 Wang <i>et al.</i> , 2015
Hami melon (<i>Cucumis melo</i>)	NO gas	- Decreases chilling injury and incidence	- Decreases the rates of O ₂ ⁻ and H ₂ O ₂ contents - Increases activity of SOD, CAT, POD and APX	Zhang <i>et al.</i> , 2017a
Highbush blueberry (<i>Vaccinium corymbosum</i>)	GSNO	- Increases firmness and postharvest life - Alleviates weight loss	- Increases ascorbate and glutathione contents	Gergoff <i>et al.</i> , 2017
Japanese plum fruit (<i>Prunus salicina</i>)	NO gas	- Delays ripening - Alleviates chilling injury symptoms during cold storage	-	Singh <i>et al.</i> , 2009
Kiwi (<i>Actinidia chinensis</i>)	NO gas/solution	- Extend postharvest life - Decreases ethylene production	- Promotes higher ascorbate content - Increases antioxidant activity - Lowers lipid peroxidation	Zhu <i>et al.</i> , 2010
Longkong (<i>Lansium domesticum</i>)	SNP	- Enhances chilling tolerance and reduces eletrolyte leakage	- Regulate the rates of O ₂ ⁻ and H ₂ O ₂ contents - Increases activity of SOD, CAT, POD and APX	Venkatachalam 2018
Mango (<i>Mangifera indica</i>)	SNP	- Increases fruit firmness - Alleviates cold damage and chilling injury - Lowers respiration peak and softening - Delays fruit color development and ripening - Increases disease resistance - Reduces the respiration rate and electrolyte leakage	- Increases activities of SOD, CAT, and POD - Lowers O ₂ ⁻ production and H ₂ O ₂ content - Increases activity of LP and PPO	Zaharah and Sing, 2011 Barman <i>et al.</i> , 2014 Hu <i>et al.</i> , 2014 Ruan <i>et al.</i> , 2015 Ren <i>et al.</i> , 2017

Papaya (<i>Carica papaya</i>)	NO gas	<ul style="list-style-type: none"> - Delays ripening and lowers softening - Lowers ethylene production, auxins, and abscisic acid content 	<ul style="list-style-type: none"> - Maintains high antioxidant capacity and total phenol and ascorbate contents 	Guo <i>et al.</i> , 2014 Lata <i>et al.</i> , 2018
Peach (<i>Prunus persica</i>)	NO gas	<ul style="list-style-type: none"> - Delays fruit senescence - Increases firmness - Increases resistance to environmental stress and disease - Lowers ethylene production 	<ul style="list-style-type: none"> - Increases levels of SOD and the enzymes of the ascorbate-glutathione cycle - Increases contents of phenolics and flavonoids 	Kang <i>et al.</i> , 2016 Li <i>et al.</i> , 2017 Han <i>et al.</i> , 2018
Peach (<i>P. persica</i>)	SNP	<ul style="list-style-type: none"> - Maintains firmness 	<ul style="list-style-type: none"> - Promotes accumulation of reactive oxygen species - Decreases the activity of SOD, CAT and POD 	Saba <i>et al.</i> , 2017 Tareen <i>et al.</i> , 2017
Tomato (<i>Solanum lycopersicum</i>)	NO/SNP	<ul style="list-style-type: none"> - Suppresses growth - Delays ripening - Increases firmness - Improves resistance to pathogenic fungi 	<ul style="list-style-type: none"> - Increases activity of SOD, CAT, POD, GST and PPO 	Eum <i>et al.</i> , 2009 Lai <i>et al.</i> , 2011 Bodanapu <i>et al.</i> , 2016
Non-Climacteric				
Chinese bayberry (<i>Myrica rubra</i>)	NO gas	<ul style="list-style-type: none"> - Affects firmness - Lowers ethylene production - Improves disease resistance 	<ul style="list-style-type: none"> - Decreases total phenolics contents and DPPH radical-scavenging activity - Delays the rates of O₂⁻ and H₂O₂ contents - Lowers lipid peroxidation - Increases activities of SOD, CAT and APX 	Wu <i>et al.</i> , 2012
Cucumber (<i>Cucumis sativus</i>)	NO gas	<ul style="list-style-type: none"> - Extends postharvest life - Improves chilling tolerance 	<ul style="list-style-type: none"> - Lowers O₂⁻ and H₂O₂ levels - Lowers lipid peroxidation - Increases activities of SOD, CAT, APX and POD 	Leshem <i>et al.</i> , 1998 Yang <i>et al.</i> , 2011
Orange (<i>Citrus sinensis</i>)	SNP	<ul style="list-style-type: none"> - Lowers weight loss - Improves resistance to fungi - Decreases chilling injury and incidence 	<ul style="list-style-type: none"> - Lowers H₂O₂ content - Increases ascorbate and phenolics contents - Increases activity of CAT, ascorbate-glutathione cycle enzymes, POD and PPO 	Mohamed <i>et al.</i> , 2016 Zhou <i>et al.</i> , 2016 Ghorbani <i>et al.</i> , 2018
Strawberry (<i>Fragaria ananassa</i>)	NO gas/DETANO/SNP	<ul style="list-style-type: none"> - Extends postharvest life - Lowers ethylene production and respiration rate 	-	Leshem and Pinchasov, 2000 Wills <i>et al.</i> , 2000, 2007 Zhu and Zhou, 2007
Sweet pepper (<i>Capsicum annuum</i>)	NO gas	<ul style="list-style-type: none"> - Delays fruit ripening 	<ul style="list-style-type: none"> - Increases ascorbate and glutathione contents and APX activity 	Chaki <i>et al.</i> , 2015 González-Gordo <i>et</i>

			<ul style="list-style-type: none"> - Enhances gene expression and enzyme activity of GalLDH - Lowers protein nitration 	<i>al., 2019</i>
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APX, ascorbate peroxidase; CAT, catalase; DETANO, Diethylenetriamine-nitric oxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GalLDH, L-galactono-1,4-lactone dehydrogenase; GSNO, nitrosogluthathione; GST, glutathione-S-transferase; LP, lipoxigenase; POD, peroxidase; PPO, polyphenol oxidase; SNP, sodium nitroprusside; SOD, superoxide dismutase.

Table 2. Interaction of NO and the defense responses of some fruits

Plant species	Pathogen	Treatment	Response/Effect	References
Climacteric				
Mango (<i>Mangifera indica</i>)	<i>C. gloeosporioides</i>	- SNP	- Reduces disease incidence and lesion diameter - Increases activity of PAL, 4CL, POD, PPO, CHI, GLU - Increases contents of phenolics and flavonoids	Hu <i>et al.</i> , 2014
Peach (<i>Prunus persica</i>)	<i>Penicillium exoansum</i>	- YS	- Reduces decay incidence - Increases total phenolics and activities of CHI, GLU, PAL, and POD - Increases NO levels	Yu <i>et al.</i> , 2012
Peach (<i>P. persica</i>)	<i>Monilinia fructicola</i>	- NO	- Induces high levels of endogenous NO, superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and NADPH oxidase - NO increases activity of NOS, CHI, GLU, PPO, and PAL, CHS, CHIS, C4H, 4CL, PR-1, PR-10 - Increases contents of phenolics and flavonoids - Salicylic acid and jasmonic acid accumulate during storage. - Enhances resistance to pathogen	Gu <i>et al.</i> , 2014 Shi <i>et al.</i> , 2015 Li <i>et al.</i> , 2017
Tomato (<i>Lycopersicon esculentum</i>)	<i>Botrytis cinerea</i>	- SNP and U0126 (MAPK inhibitor)	- NO enhances disease resistance against <i>B. cinerea</i> - NO suppresses ethylene biosynthesis - NO increases activity of NOS, CHI, GLU, PPO, and PAL - Antagonic effects of NO and U0126	Lai <i>et al.</i> , 2011 Zheng <i>et al.</i> , 2014, 2015
Tomato (<i>L. esculentum</i>)	<i>B. cinerea</i>	- L-arginine and MS	- Alleviate the incidence and severity of fruit disease - Enhance activity of SOD, CAT and POD, PR-1, PPO, PAL, GLU, and CHI - Increase NO levels	Zheng <i>et al.</i> , 2011 Zhang <i>et al.</i> , 2017
Tomato (<i>L. esculentum</i>)	<i>Rhizopus nigricans</i>	- H_2O_2 and SNP	- Enhances disease resistance - Both increase activities of PAL, CHI, GST and promotes changes in the profile of SOD, CAT and APX	Fan <i>et al.</i> , 2008
Non-Climacteric				
Citrus (<i>Citrus sinensis</i>)	<i>Colletotrichum gloeosporioides</i>	- SNP	- Reduces disease incidence and lesion diameter - Regulates hydrogen peroxide levels, stimulate the synthesis of phenolic compounds, and induce PAL, POD, PPO, CAT and, and the ascorbate-glutathione cycle	Zhou <i>et al.</i> , 2016
Pitaya (<i>Hylocereus undatus</i>)	<i>C. gloeosporioides</i>	- SNP	- Reduces disease incidence and lesion diameter - Increases activity of PAL, 4CL, POD, PPO, CHI, GLU - Increases contents of phenolics and flavonoids	Hu <i>et al.</i> , 2019

APX, ascorbate peroxidase; CAT, catalase; 4CH, cinnamate-4-hydroxylase; CHI, chitinase; CHIS, chalcone isomerase CHS, chalcone synthase; 4CL, 4-coumaroyl-CoA ligase; GLU, beta-1,3-glucanases; MAPK, mitogen-activated protein kinases; MS, methyl salicylate; POD, peroxidase; PPO, polyphenol oxidase; PR-1, PR-10, pathogenesis-related proteins 1 and 10; SNP, sodium nitroprusside; SOD, superoxide dismutase; U0126 (1,4-diamino-2,3-dicyano-1,4-bis(o - aminophenylmercapto)butadiene); YS, yeast saccharide.

Figure 1. Ethylene biosynthesis pathway and methionine recycling in plants. Ethylene is synthesized in a two-step pathway which involves the enzymes ACC synthase and ACC oxidase. The S-Adenosyl methionine (SAM) is continuously replaced by a methionine cycle (Yang cycle), although SAM can also be derived to the biosynthesis of the polyamines spermidine and spermine. Nitric oxide (NO) inhibits the ACC synthase and ACC oxidase activity, and it also affects the SAM synthetase, also known as methionine adenosyltransferases (MAT), by a S-nitrosation event. On the other hand, melatonin can stimulate the biosynthesis of NO. ACC, 1-aminocyclopropane-1-carboxylic acid; MTA, 5-methylthioadenosine; MTR, 5-methylthioribose; KMB, 2-keto-4-methylthiobutyrate.

Figure 2. Metabolism of NO in fruits. The levels of internal NO can be modified by either (A) subjecting fruits to high exogenous NO levels, or (B) stimulating the endogenous NO production by the use of L-arginine, methyl salicylate (MeSa), the elicitor of *Botrytis cinerea* (B.c.) or yeast saccharide (YS). (C) NO, which is in a higher concentration in immature green fruits than in red ripe, can be driven to protein Tyr-nitration and S-nitrosation processes which are increased at ripening. Besides, nitro-alkylation of proteins mediated by fatty acids (FA) may occur as reported elsewhere. In either green or red fruits, NO can also participate in signaling trials.