REVIEW PAPER

Interactions of melatonin, reactive oxygen species, and nitric oxide during fruit ripening: an update and prospective view

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Abstract

Fruit ripening is a physiological process that involves a complex network of signaling molecules that act as switches to activate or deactivate certain metabolic pathways at different levels, not only by regulating gene and protein expression but also through post-translational modifications of the involved proteins. Ethylene is the distinctive molecule that regulates the ripening of fruits, which can be classified as climacteric or non-climacteric according to whether or not, respectively, they are dependent on this phytohormone. However, in recent years it has been found that other molecules with signaling potential also exert regulatory roles, not only individually but also as a result of interactions among them. These observations imply the existence of mutual and hierarchical regulations that sometimes make it difficult to identify the initial triggering event. Among these ‘new’ molecules, hydrogen peroxide, nitric oxide, and melatonin have been highlighted as prominent. This review provides a comprehensive outline of the relevance of these molecules in the fruit ripening process and the complex network of the known interactions among them.

Keywords: Hydrogen peroxide, nitric oxide, nitrosomelatonin, melatonin, postharvest, ripening.

Introduction

Fruits are specialized organs whose function is to provide an appropriate environment for the formation and maturation of seeds that will be propagated by various procedures to preserve the species (Dardick and Callahan, 2014). Regardless of the different classifications of fruits, their ripening involves complex physiological processes that are associated with multiple changes at the genetic, proteomic, biochemical, and metabolic levels, which are highly coordinated (Klee and Giovannoni, 2011; Palma et al., 2011; Karlova et al., 2014; Lü et al., 2018; Palma et al., 2019; Aghdam et al., 2020b). Fleshy fruits are a good example in which endogenous metabolic fluctuations can be translated into external changes that are easily observed at the phenotypic level, in many cases consisting of drastic changes in organoleptic features (e.g. color, emission of volatiles, and flavor) (Lalel et al., 2003; Wu et al., 2018).
Plants contain a wide variety of molecules that exert regulatory functions either independently or through their interactions with other molecules, acting as plant hormones. Among the classical phytohormones are auxins, cytokinins, gibberellins (GA), abscisic acid (ABA), and ethylene, but there are also other groups, including brassinosteroids (BR), salicylates, jasmonates, and strigolactones (Vanstraalen and Benková, 2012; Asgher et al., 2017). Recently, different types of molecules previously considered toxic to cells have been found to exert signaling functions either directly or indirectly. Accordingly, molecules such as hydrogen peroxide ($\text{H}_2\text{O}_2$) and nitric oxide (NO), which are part of the metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS), have also been shown to be regulators of plant cellular metabolism, participating in all stages of plant development including seed germination, root and plant development, stomatal movement, senescence, flowering, and fruit ripening, as well as in the mechanisms of response to adverse environmental conditions (Smirnoff and Arnaud, 2019; Liu et al., 2020; Rodrigues and Shan, 2021; Corpas et al., 2022; Gupta et al., 2022). Other molecules could also be placed in the same category, such as hydrogen sulfide ($\text{H}_2\text{S}$), which has recently been shown to exert regulatory functions in numerous processes, including fruit ripening (Gotor et al., 2019; Corpas, 2019; Corpas et al., 2021; Mishra et al., 2021).

The hormone melatonin, which was discovered in higher plants in 1995 (Dubbels et al., 1995; Hattori et al., 1995), is a well-known regulatory molecule in mammals; in humans, for example, it influences numerous physiological and pathological processes such as circadian rhythms (Domínguez-Rodríguez et al., 2010), metabolism (Korkmaz et al., 2009), aging (Majidinia et al., 2018), neurodegenerative diseases (Shukla et al., 2019), and a wide range of cancers (González-Gordo et al., 2021; Moloudizargari et al., 2021). Melatonin also has a broad spectrum of functions in higher plants (Zhao et al., 2021; Arnão et al., 2022; Hernández-Ruiz et al., 2022). Consequently, melatonin has been defined as a ‘master regulator’ in animal and plant cells (Reiter et al., 2010; Wang et al., 2018; Arnão and Hernández-Ruiz, 2019; 2021a; C. Sun et al., 2021), although the available information on melatonin in plants is still limited in comparison to that in animals. Figure 1 shows a Venn diagram analysis of the number of publications from the 1980s to date covering the different signaling molecules (NO, $\text{H}_2\text{O}_2$, $\text{H}_2\text{S}$, and melatonin) as they relate to fruit physiology, with NO having the greatest number of publications, followed by $\text{H}_2\text{O}_2$, $\text{H}_2\text{S}$, and melatonin. A small number of publications have simultaneously analyzed melatonin in combination with other signaling molecules, indicating that this is an emerging area that should be addressed.

The present review provides a framework of the relevance of melatonin in fruit ripening from the perspective of $\text{H}_2\text{O}_2$ and NO metabolism. Thus, melatonin, as a free radical scavenger, exerts regulatory actions over some ROS and RNS. The biochemical interactions among melatonin and these reactive species provide a promising new area of research due to the potential signaling functions of these interactions. Furthermore, the biotechnological significance of these molecules during fruit postharvest storage is discussed, as well as the effects of their exogenous application.

**Fleshy fruit ripening**

Fleshy fruits are classically divided into two main categories according to their dependence on the ethylene profile and the respiratory burst: climacteric (e.g. apple, apricot, avocado, banana, melon, pear, persimmon, and tomato), which are dependent on ethylene and the respiratory burst, and non-climacteric (e.g. cherry, grape, orange, lemon, other citrus, olive, pepper, raspberry, and strawberry), which are not dependent on these factors (Cherian et al., 2014; Chen et al., 2018). However, fruit ripening also involves the integration of other molecules, including abscisic acid, auxin, jasmonic acid, and salicylic acid, which exert regulatory functions (Symons et al., 2012; Kumar et al., 2014; Hou et al., 2018; Kou et al., 2021; P. Li et al., 2021; Alferez et al., 2021). Recently, it was found that fruit ripening involves a physiological nitro-oxidative stress, which influences many subcellular processes such that some metabolic pathways are down-regulated whereas others are up-regulated (Chaki et al., 2015; Rodríguez-Ruiz et al., 2017; Corpas et al., 2018a; Chu-Puga et al., 2019; Palma et al., 2019; González-Gordo et al., 2019; Zuccarelli et al., 2021). Furthermore, accumulating data indicate that the exogenous application of some key molecules, such as NO, $\text{H}_2\text{O}_2$, or $\text{H}_2\text{S}$, and most recently melatonin, has beneficial effects at different levels, including delay of fruit senescence, palliating chilling injury, ameliorating fungal decay, and improving nutritional quality. In many cases, all these molecules participate in complex signaling cascades that, in general, stimulate the enzymatic and non-enzymatic antioxidant systems.

**Melatonin**

Melatonin (N-acetyl-5-methoxytryptamine) is generated from the amino acid tryptophan. In vertebrates, melatonin is the main secretory product of the pineal gland located in the brain.
and is probably best known for its influence on sleep. However, melatonin has multiple regulatory functions in physiological and pathological conditions (Shukla et al., 2019; Ma et al., 2020; Back, 2021). In plant cells, this molecule is referred to as phytomelatonin and it has phytohormonal actions in higher plants, which include its antioxidant properties. A putative melatonin receptor in the plasma membrane designated CAND2/PMTR1 (Candidate G-protein coupled receptor 2/Phytomelatonin receptor 1), which participates in the signaling mechanisms related to stomatal closure of Arabidopsis thaliana, has been identified. This signaling involves a cascade of signals, including H$_2$O$_2$, Ca$^{2+}$ influx, and K$^+$ efflux, in stomatal guard cells (Wei et al., 2018). More recently, new data obtained using confocal microscopy and CAND2-defective Arabidopsis mutants indicate that the CAND2 protein is actually localized in the cytosol and may not be a G protein that mediates melatonin-induced defense (Back and Lee, 2020). Therefore, the presence of a melatonin receptor on the plasma membrane in higher plant cells obviously remains an open question. It is, however, well recognized in higher plants that melatonin participates in regulatory functions at different levels, such as promoting lateral root growth, delaying senescence, flowering, and fruit ripening, ameliorating iron deficiency, and mediating the response to environmental stresses (Korkmaz et al., 2014; Zhang et al., 2015; Zhu et al., 2019; Zhao et al., 2019; Arnao and Hernández-Ruiz, 2020; Siddiqui et al., 2020), and that these regulatory processes involve molecules such as H$_2$O$_2$, NO, or H$_2$S that have signaling properties, although the existence of a receptor is still under analysis (S. Li et al., 2021b; Pardo-Hernández et al., 2021; Singh et al., 2022).

### Reactive oxygen species: H$_2$O$_2$ as a signal molecule

ROS are a family of molecules generated during the reduction of molecular oxygen. They include hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^-$), hydroxyl radical (•OH), and other species that do not involve electron gains, such as singlet oxygen (^1O$_2$). In higher plants, the main sources of ROS are the electron transport chains of chloroplasts and mitochondria, as well as peroxisomes, which are a particularly important source of H$_2$O$_2$ due to the β-oxidation and photosynthesis pathways (Corpas et al., 2020). Additionally, there are other minor cellular sites of ROS production including the cytosol, plasma membrane, and cell wall (Corpas et al., 2015; Podgórska et al., 2017; Kámán-Tóth et al., 2019). Furthermore, the uncontrolled overproduction of ROS, such as occurs as a consequence of adverse environmental conditions, can trigger oxidative damage to the various cellular macromolecules, causing their dysfunction (Moller et al., 2007). However, some ROS have signaling properties, in particular H$_2$O$_2$ which has been extensively studied (Exposito-Rodriguez et al., 2017; Foyer, 2018, 2020; Smirnoff and Arnaud, 2019; Nazir et al., 2020; Liu et al., 2021; Zhang et al., 2021; Zentgraf et al., 2022). Recent reports have identified two H$_2$O$_2$ plasma membrane receptors, designated leucine-rich repeat (LRR) receptor protein kinase HPCA1 (Wu et al., 2020) and LRR-receptor-like kinase (RLK) protein HSL3 (Liu et al., 2020), which sense the apoplastic content of H$_2$O$_2$ and initiate a cascade of signals as a response mechanism to different exogenous stimuli (Foyer et al., 2020; Singh et al., 2022). Likewise, during the plant immune response, O$_2^•−$ generation is controlled by a receptor-like cytoplasmic kinase (RLCK)-mediated phosphorylation of respiratory burst oxidase homolog D (RBOHD) (P. Li et al., 2021a; Singh et al., 2022).

### Reactive nitrogen species: nitric oxide as a signal molecule

The discovery that plant cells have the capacity to generate the free radical nitric oxide (NO) opened a new area of research (Kolbert et al., 2019). Unlike animals, in which the enzymatic NO source from the amino acid l-arginine involves a group of well-characterized enzymes named nitric oxide synthases (NOSs), in higher plants, the enzymatic source remains undefined, although it is generally accepted that there are two main routes: (i) a reductive pathway from nitrate and nitrite that is mediated by nitrate reductase (NR), and (ii) an oxidative pathway from l-arginine through a NOS-like activity, designated thus because it has the same biochemical requirements as animal NOS (Astier et al., 2018; Corpas et al., 2022). In higher plants, the main NO sources are the cytosol, peroxisomes, chloroplasts, and mitochondria.

Metabolism of NO leads to the formation of derived molecules, called RNS, which include nitrogen dioxide (NO$_2$), peroxynitrite (ONOO$^-$), and S-nitrosoglutathione (GSNO), among others (Corpas, 2017). ONOO$^-$ is a highly reactive molecule and also a strong oxidizing and nitrating agent (Ferrer-Sueta et al., 2018) that is formed by the chemical reaction between two radicals, NO and O$_2^•−$, with a very high rate constant (~10$^{10}$ M$^{-1}$ s$^{-1}$), even higher than the rate constant for O$_2^•−$ dismutation by the CuZn superoxide dismutase (SOD) enzyme, which is 2 × 10$^{9}$ M$^{-1}$ s$^{-1}$ (Gray and Carmichael, 1992). This characteristic guarantees that when both radicals are simultaneously present in any plant subcellular location, ONOO$^-$ will be generated. On the other hand, GSNO is also generated by the binding of NO to the thiol group of the reduced form of glutathione (GSH, γ-l-glutamyl-l-cysteinylglycine), which is considered to be the main S-nitrosothiol in plant cells whose content is regulated by the enzyme GSNO reductase (Lee et al., 2008; Corpas et al., 2013). These two examples, ONOO$^-$ and GSNO, demonstrate the close relationship between ROS and RNS metabolism.

In plants, RNS regulate protein functions by post-translational modifications (PTMs). Tyrosine nitration is an irreversible process that usually causes inhibition of the target proteins...
Compounds was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and all the hydroxy-, nitro-, and nitrosomelatonin derivatives were found.

Byeon et al. (2015) performed a systematic analysis using a LC-MS/MS approach to evaluate the content of melatonin and some of its hydroxy-derived molecules in 24 plant species. This study found that in most plant species the melatonin concentration is ~1 ng g⁻¹ fresh weight (FW). In contrast, the content of 2-OHM is ~6 ng g⁻¹ FW. A deeper analysis of the hydroxyl forms of melatonin in the selected plants indicated that the predominant form was 2-OHM (99%), followed by 4-OHM (~0.5%), with 6-hydroxymelatonin being undetected. Unfortunately, any melatonin molecule related to NO was not analyzed in this study.

Melatonin is known to react with peroxynitrite, and it could be expected that in a cellular environment, this would be a mechanism of protection against protein nitration processes that are usually associated with a down-regulation in the function of the affected protein (Begara-Morales et al., 2013; Radi, 2013; Ferrer-Sueta et al., 2018; Muñoz-Vargas et al., 2018; Corpas et al., 2021). It is important to consider that the formation of peroxynitrite is usually associated with the overproduction of both NO and O₂⁻, whose coupling activity is very high; as a result, the product, peroxynitrite, has major negative effects where it is generated. Thus, the interaction of peroxynitrite with melatonin is an additional mechanism of protection of proteins against nitration, particularly under stress conditions; this deserves to be further investigated.

Interaction among N-nitrosomelatonin, NO, and S-nitrosothiols

Early in vitro studies evaluated the capacity of NOMel to release NO. Subsequently, these assays were completed under physiological conditions where NOMel, in the presence of reducing compounds such as ascorbate, released NO and melatonin (De Biase et al., 2005). The physiological relevance of this process is similar to that exerted by S-nitrosothiols of low and high molecular weight, including GSNO, nitrosocysteine, and S-nitrosated proteins, which are also NO-releasing compounds. For example, in the presence of reductants (ascorbate and GSH, and Cu²⁺), GSNO decomposes to produce 'NO and oxidized glutathione (Gorren et al. 1996; Noble et al., 1999; Holmes and Williams 2000; Smith and Dasgupta 2000). In 30-day-old Arabidopsis plants, exogenous GSNO applied to the root system was available to release NO and mediate up to 1945 genes that were expressed differently in leaves and roots, with 114 genes being exclusive to one of these organs, indicating the capacity of the GSNO to move long distances through the vascular system (Begara-Morales et al., 2014b). Based on this property, Fig. 3 shows a working model in which NOMel could release NO and mediate a process of S-nitrosation of GSH, free cysteine, and thiol groups of proteins. At the same time, these S-nitrosothiols release NO and, by a trans-nitrosation process,
mediate the formation of NOMel (Peyrot et al., 2006; Berchner-Pfannschmidt et al., 2008; Hickok et al., 2012; Mukherjee, 2019; Hardeland, 2021). These mechanisms could be considered a cellular strategy to extend the functional actions of the involved molecules in the different subcellular compartments in which they are generated. Consequently, a close relationship among all these molecules with the capacity to carry and release NO should be anticipated, with this being a long-distance signaling mechanism (Singh et al., 2016).

In higher plants, the information about the formation of hydroxy- and nitromelatonin metabolites is, to the best of our knowledge, limited, and it is mostly based on in vitro and in vivo studies of animal cells (Hardeland, 2021). However, it could be expected that these compounds should have analogous functions in plant cells. In addition to the direct interactions between melatonin and the different ROS and RNS, there are also some mechanisms in plants that mediate the conversion of melatonin into 2-OHM and cyclic 3-hydroxymelatonin.
through the enzymatic action of melatonin 2-hydroxylase (M2H) and melatonin 3-hydroxylase (M3H), respectively (Lee and Back, 2016; Lee et al., 2016). Thus, some genetic studies in rice plants using RNA interference approaches to down-regulate the expression of M2H caused an increase in the content of melatonin, conferring a higher tolerance to diverse stresses including cadmium, senescence, salt, and tunicamycin (Choi and Back, 2019). Similarly, treatment with 2-OHM induced plant defense genes in Arabidopsis, although to a smaller extent than melatonin (Byeon et al., 2015), and in rice (Oryza sativa) 2-OHM triggered resistance against cold and drought stress (Lee and Back, 2016, 2019).

In cassava (Manihot esculenta) plants, there are obvious protein interactions among cytosolic ascorbate peroxidase (MeAPX2) and two cytosolic isozymes of the melatonin biosynthesis, tryptophan decarboxylase (MeTDC2) and N-acylserotonin O-methyltransferase (MeASMT2), which provide a higher antioxidant capacity against H₂O₂ (Bai et al., 2020). This observation prompts several questions focused on the molecular mechanism underlying how these protein interactions (MeAPX2–MeTDC2 and MeAPX2–MeASMT2) occur, where they take place, and whether they increase the APX activity and therefore provide greater protection against high concentrations of H₂O₂.

In higher plants, some experimental studies have reported an interaction between melatonin and NO (Y. Sun et al., 2021). In sunflower (Helianthus annuus L.) seedlings subjected to salinity stress, the exogenous application of 15 µM melatonin altered the content of NO, O₂⁻, and ONOO⁻, and consequently the modulation of CuZn-SOD and Mn-SOD as well as protein tyrosine nitration (Arora and Bhatla, 2017). Recently, using 3-day-old Arabidopsis seedlings as a model, Singh et al. (2021) estimated the NO release capacity of two compounds, 250 µM GSNO and NOMel, applied through the root system, and evaluated the NO content in green cotyledons by confocal laser scanning microscopy. The results showed that NOMel is more efficient than GSNO in releasing NO. Consequently, these data indicate that both NOMel and GSNO have the capacity to travel through the vascular system and release NO in other parts of the plant, as was previously proposed (Airaki et al., 2011; Begara-Morales et al., 2014b; Singh et al., 2016).

Crosstalk among melatonin, H₂O₂, and NO in fruit ripening and postharvest storage

Knowledge of the mechanism of regulation among melatonin, NO and H₂O₂ during fruit ripening is in a nascent phase, especially due to the fact that there are earlier unresolved issues such as the identity of the genes involved in the melatonin biosynthesis pathway, as well as how the NO is generated. Since the first descriptions of the presence of melatonin in plants (Dubbels et al., 1995; Hattori et al., 1995), interest in this molecule in the field of plant physiology has grown exponentially. This is especially due to its antioxidant properties, as well as its regulatory functions affecting both gene and protein expression, enzyme activities, and their crosstalk with different phytohormones (Arnao and Hernández-Ruiz, 2021b; Arnao et al., 2022). Likewise, the study of the interactions of endogenous melatonin with ROS and RNS in higher plants has also been increasing, mainly based on the biochemical information established from animal studies that have provided basic knowledge in this field. However, research studies of the interactions between melatonin with both ROS and RNS during fruit ripening are still scarce (Aghdam et al., 2022), possibly due to the low levels of endogenous melatonin in fruits, which make it difficult to identify the related melatonin metabolites. For example, a comparative analysis of the melatonin content of the most consumed horticultural fruits worldwide, pepper (Capsicum annum L.) and tomato (Solanum lycopersicum L.), which are representative examples of non-climacteric and climacteric fruits, respectively, indicated that the melatonin concentration in red pepper fruits of six cultivars ranged from 5 ng g⁻¹ to 12 ng g⁻¹ FW, whereas in red tomato fruits of seven cultivars, the melatonin concentration ranged from 0.6 ng g⁻¹ to 15 ng g⁻¹ FW (Riga et al., 2014). A similar situation is apparent concerning studies on the metabolism of endogenous NO in fruits, about which information is also very limited (Corpas et al., 2018a), although in the case of ROS metabolism there is more information available.

Other challenges in delving into the regulatory mechanisms at the genetic level are to identify all the genes/enzymes involved in melatonin and NO biosynthesis. In the case of melatonin, its synthesis from the amino acid tryptophan in

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**Fig. 3.** Simple model of melatonin (Mel) nitrosation, S-nitrosation of glutathione (GSH), cysteine (Cys), or protein thiol (P-SH), and trans-nitrosation. Nitric oxide (NO) interacts with Mel, GSH, Cys, and P-SH to generate nitrosomelatonin (NOMel), S-nitrosogluthathione (GSNO), S-nitrosocysteine (CysNO), or nitrosated protein (P-SNO), respectively, which can undergo trans-nitrosation processes.
| Fruit          | Concentration | Main effects                                                                                                                                                                                                 | Reference                        |
|---------------|---------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| Melatonin     |               | **Peach** *(Prunus persica L.)* 0.1 mM Delays postharvest senescence by lowering O$_2^•$ and H$_2$O$_2$ accumulation. Higher AA accumulation and increased activity of catalase, SOD, and APX | Gao et al., (2016)               |
|               |               | **Grapevine** *(Vitis vinifera × labrusca)* 0.2 mM Stimulates ripening by increasing the levels of ABA, H$_2$O$_2$, and ethylene                                                                                       | Xu et al., (2018)                |
|               |               | **Pear** *(Pyrus communis L.)* 0.1 mM Delays postharvest senescence and induces NO accumulation. Higher NOS-like gene expression and enzyme activity. Lower ACS, ACO, PG, and Cel genes expression | Liu et al., (2019)               |
|               |               | **Pear** *(Pyrus communis L.)* 0.1 mM Induces anthocyanin accumulation through the H$_2$O$_2$ generated by RBOHF                                                                                               | H. Sun et al., (2021)            |
|               |               | **Sweet cherry** *(Prunus avium L.)* 0.1 mM Higher endogenous melatonin accumulation. Higher SOD, CAT, APX, and GR enzyme activity. Higher ascorbate and GSH accumulation. Lower membrane integrity. Lower electrolyte leakage and MDA accumulation. Lower O$_2^•$ and H$_2$O$_2$ accumulation | Wang et al., (2019)              |
|               |               | **Sweet cherry** *(Prunus avium L. var Prime Giant)* 0.01 and 0.1 mM Delays ripening by modulating the contents of endogenous hormones, mainly ABA and auxin | Tijero et al., (2019)            |
|               |               | **Sweet cherry** *(Prunus avium L.)* 0.50 and 0.1 mM Treatment of leaves treated with melatonin improved the antioxidant content of sweet cherry fruit                                                               | Xia et al., (2020)               |
|               |               | **Jujiube** *(Ziziphus jujuba Mill.)* 25 µM Higher APX and GR enzyme activity. Higher ascorbate and GSH accumulation. Lower PG and PME enzymes activity, maintaining firmness | Tang et al., (2020)              |
|               |               | **Pomegranate** *(Punica granatum L.)* 0.1 mM Higher NADPH accumulation. Higher APX, GR, G6PDH, 6PGDH, and PAL enzyme activity. Higher AOX gene expression. Higher phenol and anthocyanin accumulation and DPPH-scavenging capacity. Higher AA and GSH accumulation. Lower AAO enzyme activity. | Aghdam et al., (2020a)           |
|               |               | **Mango** *(Mangifera indica L.)* 0.2 mM Delays the ripening process. Decreases the contents of H$_2$O$_2$ and MDA in the exocarp of the fruit                                                                                 | Dong et al., (2021)              |
|               |               | **Apple** *(Malus domestica L. Borkh)* 1 mM Reduces ethylene production. Increase the activity of catalase, SOD and peroxidase and keeps apple quality during postharvest storage | Onik et al., (2021)              |
|               |               | **Blueberry** *(Vaccinium corymbosum L.)* 1 mM Reduces qualitative decay and improves antioxidant system (catalase, SOD, APX, ascorbate, polyphenols, anthocyanins, and flavonoids) during cold storage | Magri and Petriccione, (2022)    |
|               |               | **Kiwifruit** *(Actinidia chinensis)* 0.1 mM Palliates chilling injury during cold postharvest storage by inhibition of lignin metabolism and increasing the activity of antioxidant enzymes and the content of soluble antioxidants (ascorbate and GSH) | Jiao et al., (2022)              |
|               |               | **Tomato** *(Solanum lycopersicum)* 0.5 mM Promotes ripening of postharvest fruit through DNA methylation of ethylene-signalling genes                                                                                | Shan et al., (2022)              |
| H$_2$O$_2$    |               | **Melon** *(Cucumis melo L.)* 20 mM Treatment of melon plants increases the soluble sugar content in leaves and fruits, thus improving the fruit quality. Increases photosynthetic activity and the activities of chloroplastic and cytosolic fructose-1,6-bisphosphatase, sucrose phosphate synthase, and inverases | Ozaki et al., (2009)             |
|               |               | **Longan** *(Dimocarpus longan Lour)* 1.96 mM Increases the activities of pulp PLD, lipase, and LOX. Destroys longan pulp membrane structure and increases cell membrane permeability | Lin et al., (2019)               |
|               |               | **Guava** *(Psidium guajava L.)* 250 mM Reduces enzymatic browning of freshly cut fruit by reducing PPO and POD activities. Stimulates the peroxiredoxin/thioredoxin system | Chumyam et al., (2019)           |
higher plants seems to involve six enzymes that are present in different subcellular compartments, namely tryptophan de-carboxylase (TDC), tryptamine 5-hydroxylase (T5H), tryptophan hydroxylase (TPH), serotonin N-acetyltransferase (SNAT), N-acetylserotonin methyltransferase (ASMT), and cafféic acid O-methyltransferase (COMT). However, not all genes/enzymes have been identified in all plants (Aghdam et al., 2022 and references therein) suggesting the existence of diverse biosynthesis pathways. In the case of NO, its biosynthetic pathway is even more disputed (Corpas et al., 2022 and references therein). It would be of great interest if any of the enzymes involved in melatonin biosynthesis were found to be targets of NO-derived PTMs such as S-nitrosation and nitration, although, to our knowledge, this information has not yet been uncovered.

Consequently, the majority of the studies on fruits have been carried out after the exogenous application of either melatonin, NO, or H₂O₂. The few results reported indicate that these molecules regulate the ripening process, either slowing or accelerating it, or provide beneficial effects during postharvest storage; as a result, these compounds could be used as tools for biotechnological approaches to maintain the quality of the fruits as well as protecting them against infections by pathogens or chilling damage associated with postharvest storage. It should be pointed out, however, that the effects of these molecules on fruit ripening depend on the type of fruit (climacteric or non-climacteric) and the dose and duration of the treatment, among other parameters that must be optimized. Table 1 summarizes representative examples of climacteric and non-climacteric fruits treated with melatonin, H₂O₂, or NO and the beneficial effects of these treatments, such as extending postharvest storage life or preserving nutritional quality. However, it seems evident that the metabolic triangle constituted by melatonin, NO, and H₂O₂ has a common characteristic that implies the activation of both enzymatic and non-enzymatic antioxidant systems (Tan et al., 2015; Guo et al., 2020).

### Table 1. Continued

| Fruit                      | Concentration | Main effects                                                                 | Reference        |
|----------------------------|---------------|-------------------------------------------------------------------------------|------------------|
| Kyoho grape                | 300 mM        | Promotes early ripening. Affects the gene expression of HSP, GDSL, XTH, and CAB1, involved in oxidative stress, cell wall deacetylation, cell wall degradation, and photosynthesis, respectively. | Guo et al., (2020) |
| (Vitis vinifera x Vitis labrusca) |               |                                                                               |                  |
| Mango                      | 20 mM         | Treated mango plants have fruits with a higher content of total sugar, phenol, and carotenoids | Mostafa, (2021)  |
| (Mangifera indica L.)      | 100 mM        | Increases tomato fruit firmness, decreases water-soluble pectin and expression of cell-wall-related genes, polygalacturonase, and pectate lyase. Maintains morphological and biochemical quality of tomato fruits during postharvest storage | Torun and Uluisik, (2022) |
| Tomato                     | 10 ppm NO gas | Retards cell wall degradation                                                 | Zhao et al., (2019) |
| (Solanum lycopersicum L. cv. Verty F₁) | 20 ppm NO gas | Promotes ascorbate biosynthesis and intensifies protein nitration, and S-nitration. Decreases catalase activity and APX activities | Rodriguez-Ruiz et al., (2017); González-Gordo et al., (2019) |
| Strawberry                 | 5 µM sodium nitroprusside solution | Extends postharvest life | Wills et al., (2007); Zhu and Zhou, (2007) |
| (Fragaria x ananassa Duch.) |               |                                                                               |                  |
| Peach fruit                | 10 ppm NO gas | Delays the ripening process. Affects sucrose metabolism by changing the expression of related genes | Han et al., (2018) |
| (Prunus persica L. cv. Xiahui 6) |             |                                                                               |                  |
| Jujube                     | 20 ppm NO gas | Retards cell wall degradation                                                 | Zhao et al., (2019) |
| (Ziziphus jujuba Mill.)    | 5 ppm NO gas  | Delays fruit ripening. Increases ascorbate content, protein nitration, and S-nitration. Decreases catalase activity and APX activities | Rodriguez-Ruiz et al., (2017); González-Gordo et al., (2019) |
| Sweet pepper               | 10 ppm NO gas | Promotes ascorbate biosynthesis and intensifies protein nitration, and S-nitration. Decreases catalase activity and APX activities | Rodriguez-Ruiz et al., (2017); González-Gordo et al., (2019) |
| (Capsicum annuum L. cv. Melchor) | 20 ppm NO gas | Promotes ascorbate biosynthesis and intensifies protein nitration, and S-nitration. Decreases catalase activity and APX activities | Rodriguez-Ruiz et al., (2017); González-Gordo et al., (2019) |
| Tomato                     | 300 ppm NO gas| Promotes ascorbate biosynthesis and intensifies protein nitration, and S-nitration. Decreases catalase activity and APX activities | Rodriguez-Ruiz et al., (2017); González-Gordo et al., (2019) |
| (Solanum lycopersicum L. cv. ‘Micro-Tom’) |            |                                                                               |                  |
| Strawberry                 | 100 ppm NO gas| Enhances postharvest disease resistance to the fungus Alternaria alternata by postponing ethylene biosynthesis | Wei et al., (2021) |
| (Capsicum melo L.)         |               |                                                                               |                  |

AA, ascorbic acid; AAO, ascorbic acid oxidase; AOX, alternative oxidase; ABA, abscisic acid; ACS, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase; ACO, ACC oxidase; APX, ascorbate peroxidase; CAB1, chlorophyll a-b binding protein; CAT, catalase; Cel, cellulose; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GDSL, GDSL-motif esterase/lipase; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GSH, reduced glutathione; HSP, heat shock protein; LOX, lipoxygenase; MDA, malondialdehyde; NOS, NO synthase; PG, polygalacturonase; 6PGDH, 6-phosphogluconate dehydrogenase; PLD, phospholipase D; POD, peroxidase; PPO, polyphenol oxidase; RBOHF, respiratory burst oxidase homolog F; SOD, superoxide dismutase; XTH, xylolucan endotransglycosylase/hydrolase.

**Table 1.** Continued.
Chumyam et al., 2019; Zuccarelli et al., 2021). This may serve to control the overproduction of ROS and RNS that could trigger uncontrolled nitro-oxidative stress resulting in an alteration in the quality of the fruits, in terms of both their external appearance and their organoleptic qualities (aroma, flavor, acidity, sweetness, etc.).

In pepper fruits, treatment with NO gas causes delayed ripening, which is accompanied by a modulation of the ROS metabolism characterized by an elevation in ascorbate content as a consequence of an increase in the expression and activity of the last enzyme of its biosynthesis pathway, the mitochondrial enzyme 1-galactono-1,4-lactone dehydrogenase (GalLDH) (Rodriguez-Ruiz et al., 2017). Likewise, the NO-treated fruits had a higher GSH content, higher APX and lipoxygenase activities, lower lipid peroxidation, and lower \( \text{O}_2^- \)-generating NADPH oxidase activity (González-Gordo et al., 2019, 2020). Interestingly, a higher content of nitrated proteins was apparent, particularly the peroxisomal enzyme catalase, whose activity decreased (Chaki et al., 2015). These observations related to APX and catalase activity are in good agreement with the previously reported effect of NO-derived PTMs, S-nitrosation, and nitration on these enzymes in other plant species (Begara-Morales et al., 2014a; Palma et al., 2020).

Similarly, the exogenous application of NO to tomato at the pre-climacteric stage suppressed the activity of antioxidant enzymes, increased protein S-nitrosation and nitration, and favored the accumulation of ascorbate and flavonoids (Zuccarelli et al., 2021). Recently, it has been shown that melatonin exerts an epigenetic regulation through DNA methylation of ethylene signaling genes, which promotes the ripening of tomato fruit during postharvest storage (Shan et al., 2022). This observation suggests a scenario to be addressed in future investigations.

The cascade of events that takes place when any of these molecules is applied exogenously has been the subject of many studies because there are other elements involved, such as the type of fruit, the involvement of phytohormones such as ethylene, or the state of preservation of the fruit, for example, at low temperature. For example, in pear fruits, the exogenous application of melatonin inhibits the synthesis of ethylene, which seems to be mediated by NO (Liu et al., 2019), since this molecule can inhibit key enzymes in the ethylene biosynthesis pathway, such as \( \text{S} \)-adenosyl methionine synthetase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and ACC oxidase (Palma et al., 2019). In the case of non-climacteric fruits, the ripening process is essentially modulated by ABA, which mediates the accumulation of anthocyanins and sugars. For example, in sweet cherry, exogenous melatonin delays fruit ripening, counteracting the effect of ABA, since it affects the balance of other involved phytohormones such as cytokinins, jasmonic acid, and salicylic acid (Tijero et al., 2019; Michailidis et al., 2021).
Conclusions and future perspectives

As in mammals, melatonin is a multifunctional molecule in higher plants and specifically in fruits, where it exerts numerous beneficial functions as a protectant against biotic and abiotic stresses when it is exogenously applied. Melatonin has antioxidant properties, since it reacts with both ROS and RNS, although the information available on the derived molecules is scarce in higher plants and even non-existent in relation to the ripening of fruits, a process that is characterized by an important nitro-oxidative metabolism.

Future research should focus on the interactions and functions of these molecules, although a major technical challenge is their identification and specific localization, considering that they are endogenously generated at very low concentrations. Unquestionably, the exogenous application of melatonin has been shown to be a powerful biotechnological tool, since it exerts beneficial effects either directly as an antioxidant molecule, or by acting as a signaling molecule that acts upstream of H2O2 and NO. Furthermore, the interaction of melatonin with NO to generate nitrosomelatonin, a molecule that can release NO in the presence of reductants such as ascorbate, opens new research lines related to the complex crosstalk between these molecules. On the other hand, the use of exogenous melatonin to provide beneficial effects during postharvest storage could be considered as a novel biotechnological tool for application in the horticultural industry. However, it is important to note that the melatonin concentration, the time of exposure, and the means of application (by immersion, spraying, or other methods) should be optimized for each type of fruit. Figure 4 illustrates the cascade of signals mediated by the crosstalk among melatonin, NO, and H2O2 during fruit ripening, which, in general, stimulates antioxidant capacity through the activation of enzymatic and non-enzymatic systems as well as triggering regulatory functions in gene regulation by their interactions with the different phytohormones. Consequently, we conclude that melatonin, besides being an antioxidant molecule, it is also a key molecule with signaling properties. Beyond scientific interest in the basic research on the complex regulatory function of melatonin and its crosstalk with NO and H2O2 during the ripening of fruits or their subsequent storage, from an anthropological point of view, one of the stimuli that may promote its use is the nutraceutical benefits fruits enriched in melatonin can provide for human health.

Conflict of interest

The authors declare no conflict of interest.

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