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Review

# Reactive oxygen species and heavy metal stress in plants: Impact on the cell wall and secondary metabolism☆

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ABSTRACT

The production of reactive oxygen species (ROS) in plants is part of the normal metabolism of chloroplasts, mitochondria and peroxisomes; however, the exposure to environmental constraints, like toxic concentrations of heavy metals, can overwhelm the systems protecting the plants and result in oxidative stress. The formation of ROS is also an event accompanying normal physiological processes, namely pollen tube growth, cell wall loosening during cell expansion, plant fibre growth, lignification, organ senescence and fruit ripening. Since reviews have already been published on broad molecular aspects underlying the production of ROS in plants as a consequence of heavy metal stress, we will here adopt a narrower angle by focusing on two specific aspects: 1) the effects of heavy metal-induced ROS on plant cell wall-related processes and 2) the stimulatory/inhibitory effects of ROS on plant secondary metabolism. We will highlight the role of ROS in important physiological processes, namely fruit maturation, bast fibre intrusive growth (where a model is proposed), pollen tube growth and as regulators of senescence. We will end our survey with an outlook on the importance of deciphering the signaling cascade underlying ROS production in response to heavy metal stress through specific comparisons. In particular, we will hint at comparative studies on 1) ancient local varieties (which often display enhanced resistance to environmental constraints, as well as high secondary metabolite contents) and commercial counterparts, 2) hyperaccumulators and normal plants from the same species. Such studies will enable a better understanding of the impact of ROS on physiological processes, namely the control of plant cell size and organs, as well as on processes of industrial interest, i.e. the production of secondary metabolites.

## 1. Introduction

Anthropogenic activities, such as industrialization and ever-increasing urbanization, are accompanied by the release of pollutants in the environment, i.e. heavy metals (metals with high atomic weight/number/density) and nanomaterials, which can in their turn cause phytotoxicity by strongly impacting plant physiology and development. Being sessile organisms, plants have evolved complex mechanisms to respond to an adverse environment which rely on stimuli perception, intracellular signaling and enzymatic/non-enzymatic responses. Heavy metal stress is a serious threat to plant growth and development and is consequently a major abiotic stress factor affecting crop yield, as well as animal/human health, once plants enter the trophic chain (Khan et al., 2015). One of the promptest effects when plant cells are exposed to toxic concentrations of heavy metals is the production of reactive oxygen species (ROS), i.e. superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($^{\bullet}OH$), as well as non-radicals, such as hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) (Smeets et al., 2005; Tamás et al., 2017). ROS can be produced either directly by ROS-active metals through the Haber-Weiss/Fenton reactions, or indirectly, by the stimulation of NADPH oxidases (NOXs), or by inhibiting enzymes through the displacement of essential cations (Shahid et al., 2014). Peroxisomes, chloroplasts and mitochondria in the first place, as well as the

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cell wall, endoplasmic reticulum and plasma membrane, are the sites where ROS are produced (Das and Roychoudhury, 2014; Kärkönen and Kuchitsu, 2015). These species, representing the by-products of the aerobic metabolism, comprise both short-lived species and members with a longer half-life (milliseconds). The former, like $O_2^{\cdot-}$ (a few microseconds), cannot diffuse through membranes because of their charge, while the latter like $H_2O_2$, are able to cross membranes via aquaporins (Bienert et al., 2007). When ROS concentrations surpass the cellular detoxification capacity, the cell enters into a state of oxidative stress measurable by the increased oxidation of molecules, such as DNA, proteins lipids and carbohydrates.

ROS are considered a "double-edged sword" in plant physiology (Mittler, 2017): on one hand, they induce oxidative damage in the tissues; on the other hand, they act as signaling molecules that partake in important plant developmental processes. There indeed exists a plethora of plant physiological processes regulated by ROS (Swanson and Gilroy, 2010), most notably polar growth (Mangano et al., 2016a), protein kinase cascades (Pitzschke and Hirt, 2009), transcriptional activities (Tripathy and Oelmüller, 2012; Xu et al., 2014) and cell wall modifications (loosening and/or stiffening) (Kärkönen and Kuchitsu, 2015; Müller et al., 2009; O'Brien et al., 2012).

A dual cellular system based on both enzymatic and non-enzymatic players scavenge ROS produced in plants (Gill and Tuteja, 2010). The first mechanism relies on the enzymes superoxide dismutase (SOD), ascorbate/guaiacol peroxidase (APX/GPX), glutathione-*S*-transferases (GST) and catalase (CAT). The second mechanism is the non-enzymatic response regulating the production of polyamines, as well as low-molecular weight compounds, such as reduced glutathione (GSH), vitamins such as ascorbic acid and α-tocopherol, osmolytes like proline and secondary metabolites, notably flavonoids, phenolics, carotenoids (Akram et al., 2017).

ROS have accompanied aerobic life from the very beginning: as recently commented in the literature, the high reducing primordial conditions on Earth and elevated soluble Fe content in the oceans resulted in the immediate conversion of oxygen into ROS (Mittler, 2017). Hence, ROS have been a hallmark of aerobic life and gone hand-in-hand with the development of a cellular system able to detoxify them and prevent oxidative stress. Given their versatility in terms of reactivity, ability or not to diffuse, sites of production, they have been "selected" as ideal plant signaling molecules monitoring different cell conditions (Mittler, 2017) (e.g. stress perception, programmed cell death, pathogen response, growth and development of tissues/organs).

In the next paragraph we will give examples of ROS as key players in plant cell physiology and discuss the implications of their action and ultimate effects.

## 2. ROS in plants: scourge or boon?

ROS are by-products of metabolic processes (where their levels are controlled by the aforementioned endogenous antioxidant system), but they are also produced in case of exogenous stresses (where their high levels can trigger irreversible processes, like cell death). Increasing data in the literature unquestionably point to a fundamental role of ROS in important developmental processes, where they function as signaling system. It has indeed been proposed to refer to the cellular components interacting with ROS as "ROS processing system" instead of "antioxidant system" (Noctor et al., 2018). The dual role of ROS, i.e. as regulatory system, as well as causal agent of oxidative damage will be hereafter discussed, by describing some key physiological processes in which ROS intervene, as well as the extreme consequences of their accumulation. The examples chosen are representative of the complexity of the role of ROS and are mostly cell wall-oriented, to provide a continuum with what discussed in the next paragraph.

### 2.1. Fruit maturation and postharvest shelf life

The production of ROS is a process accompanying climacteric fruit maturation. During peach fruit maturation, a high respiratory rate was recorded, which was accompanied by the production of ROS immediately preceding rapid expansion (Huan et al., 2016). Therefore, ROS seem to act as signaling molecules triggering fruit expansion. During this stage, the study showed that the main antioxidant enzymes are Fe-SODs, CAT2 and GPX8. However, during late stages of fruit maturation (when $H_2O_2$ levels are the highest), ROS exert oxidative damage, as revealed by high electrolyte leakage and malondialdehyde levels. At this stage, the most expressed genes involved in the antioxidant system are *CAT1* and *GPX6*. Hence, there appears to be a fine-tuned regulatory system involving several members of the same enzyme family that intervene at different stages of the fruit maturation.

In another climacteric fruit, "Golden" papaya, maturation was accompanied by an enhanced lipid peroxidation, together with a steady increase in ascorbate peroxidase and other antioxidant enzymes, notably CAT, glutathione reductase, SOD (Resende et al., 2012).

ROS scavenging activity has also been related to economically important parameters, namely fruit postharvest quality. Indeed, in tomato, the ROS-scavenging ability of flavonoids was directly correlated to postharvest shelf life: the accumulation of these secondary metabolites contributes to delay over-ripening, thereby lowering the susceptibility to *Botrytis cinerea* infection. In particular, flavonoids with 3 −OH on the B-group decrease the susceptibility to the infection, by suppressing the spread of ROS (Zhang et al., 2015).

One of the clearest examples of ROS affecting shelf life is perhaps the oxidative burst within 72 h after harvesting cassava. The cassava cytochrome *c* oxidase is cyanide sensitive, resulting in the accumulation of ROS immediately after harvest. Expression of the *Arabidopsis* alternative oxidase gene (*AOX1*) in cassava prevents the over-reduction of the mitochondrial complexes I and III, thus eliminating the accumulation of ROS (Zidenga et al., 2012). A similar prolongation of the shelf life was observed in cassava varieties producing high amounts of β-carotene (Morante et al., 2010), an antioxidant able to quench ROS.

We will also here briefly mention the role of nitric oxide (NO) in the light of its impact on fruit ripening (Manjunatha et al., 2010), as well as on important plant developmental processes, response to abiotic stresses and cross-talk with ROS (Domingos et al., 2015; Liu et al., 2018; Tripathi et al., 2017). Fruit fumigation with NO extends the shelf life of e.g. strawberry and kiwi, as well as of vegetables like broccoli and protects against phytopathogens, via a mechanism counteracting ethylene production and ROS over-accumulation. NO indeed binds to ACC (1-aminocyclopropane-1-carboxylate) oxidase, a complex then stabilized by ACC, and causes a decrease in ethylene biosynthesis (Manjunatha et al., 2010). Additionally, NO suppresses the signaling cascades of ROS and has an effect on the enzymes involved in ROS scavenging, notably SOD and CAT (Manjunatha et al., 2010).

### 2.2. Plant fibre growth

To elucidate the impact of ROS on plant fibre growth, we will here take as emblematic example cotton, which produces seed trichomes. We will additionally propose a model explaining the involvement of ROS in the development of other types of plant fibres, notably phloem fibres (a.k.a bast fibres).

The cotton fibre is an important textile material of great economic value. Fibre length and secondary wall (SCW) thickening of fibres are important indices of quality (Guo et al., 2016; Tang et al., 2014), which are mainly determined by the elongation duration and SCW biosynthesis, respectively. There is evidence that ROS are involved in both fibre elongation and SCW formation. ROS are generated during cotton fibre initiation and elongation (Mei et al., 2009). Fibre elongation requires optimal $H_2O_2$ levels. Tang and colleagues observed that blocking the activity of the NADPH oxidase with diphenyleneiodonium (DPI) inhibits ROS formation and fibre cell elongation (Tang et al., 2014), while another study identified a high accumulation of a gene encoding a cytosolic ascorbate peroxidase (*GhAPX1*) during cotton fibre elongation

likely to detoxify $H_2O_2$ (Li et al., 2007). One way in which ROS (hydroxyl radicals) regulate growth is through the scission of xyloglucan polymers, decreasing the resistance of the wall to the pressure from the expanding protoplast (Gapper and Dolan, 2006). *GhCaM7* (a gene encoding a calcium sensor) is also an important regulator of early fibre elongation by modulating ROS production (Tang et al., 2014). *GhCaM7* over-expression in cultivated cotton resulted in increased ROS concentrations in fibre cells and early fibre elongation compared with the wild type (Hande et al., 2017). Furthermore, $H_2O_2$ enhances $Ca^{2+}$ influx into the fibre and regulates the expression of *GhCaM7* (Hande et al., 2017).

Besides the positive effect on fibre elongation, a high accumulation of $H_2O_2$ acts as a signaling event for the initiation of SCW thickening (Guo et al., 2016; Tang et al., 2014). It was observed that incubating developing cotton fibres with $H_2O_2$ scavengers blocks differentiation, whereas applying $H_2O_2$ stimulates SCW formation (Gapper and Dolan, 2006). This phenomenon can be explained by the activation of the dimerization of cellulose synthase subunits via the Zn domains (intermolecular disulfide bridges) located within the cytoplasmic N-terminal region of the proteins. This event promotes the synthesis of cellulose and triggers SCW formation (Gapper and Dolan, 2006; Guo et al., 2016; Kurek et al., 2002).

Not much is available in the literature concerning the role of ROS in regulating the growth of another type of plant fibres, i.e. bast fibres. Their growth mechanism is intrusive, i.e. their pointy ends invade the middle lamellas of neighboring cells (Ageeva et al., 2005; Guerriero et al., 2014; Snegireva et al., 2010), thereby achieving noteworthy lengths (in the order of several mm in some instances). High-throughput studies using RNA-Seq have been published in flax and hemp (Gorshkov et al., 2016; Guerriero et al., 2017), but in none the role of ROS is discussed or has been reported, since the main focus was on cell wall-related processes. We would here like to propose a model on intrusive growth which takes into account ROS generation, as well as the jasmonic acid (JA)-driven wound response generated by the invasion of the middle lamellas of the surrounding parenchymatic cells (Fig. 1).

Genes involved in the synthesis of indole glucosinolates and JA were upregulated in actively elongating hemp internodes (Guerriero et al., 2017). These findings can be interpreted considering the following scenario: at the cell wall, the intrusive growth generates a response of the damage-associated molecular pattern (DAMP) type, which is transduced to the cell interior via calcium and activates genes involved in the synthesis of JA (lipid intermediates shuttling between the chloroplast and the peroxisome). Cell wall damage is known to be perceived by "sentinels", i.e. receptors which sense the cell wall integrity status (Hamann, 2015). However, it was discussed that the receptor THESEUS1, more than acting as a sentinel, could mediate the early and late cell wall damage response, by connecting with the NADPH oxidase (NOX)-induced ROS burst in the apoplast (Denness et al., 2011). In this respect it should be noted that the receptor-like kinase ANXUR was found to act upstream of NOX in the pollen tube model and to partake in the regulation of ROS production (Boisson-Dernier et al., 2013). Since intrusive growth is a mechanism that has high similarity to fungal hyphae invasion, it is plausible to assume that ROS are generated at the cell wall via NOX. However, the wound response does not lead to callose deposition (Snegireva et al., 2010), indicating that the plant is somehow able to recognize the "self" from the "non-self" (e.g. pathogens).

ROS and JA form a negative feedback loop that can suppress each other's production (Denness et al., 2011). Consequently, JA signaling (mediated by the bHLH transcriptional regulator MYC2; Dombrecht et al., 2007; Kazan and Manners, 2013) is influenced and therefore not sufficient to unleash the suite of events accompanying the canonical pathogen response mechanism.

Flavonoid biosynthetic processes were also enriched in young hemp internodes (Guerriero et al., 2017): these secondary metabolites constitute the non-enzymatic antioxidant system scavenging ROS. Their synthesis may contribute to further limit the wound response caused by elongating bast fibres. In the cortical tissues harboring the hypolignified bast fibres, lignification needs to be controlled too; therefore, the ROS generated in the neighboring cells need to be limited to avoid lignification. Indeed, flax mutants (*lbf1*; Chantreau et al., 2014) displaying ectopic lignification of the bast fibre cell walls had increased expression of NOX in the cortical tissues.

The presence of too high JA concentrations exerts inhibitory effects on cotton fibre growth (Tan et al., 2012): in bast fibres a regulatory mechanism must therefore exist (as the one controlled by the transcription factor TCP in cotton; Hao et al., 2012) which ensures an

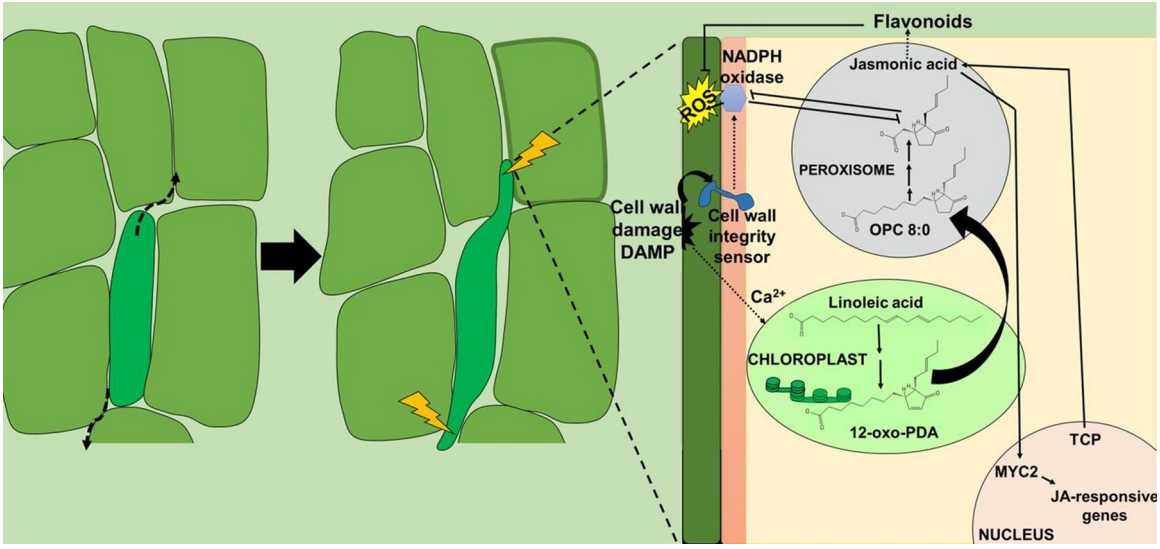


**Fig. 1.** Model depicting the role of ROS in bast fibre intrusive growth. The pointy ends of elongating bast fibres cause cell wall damage (DAMP type) in the neighboring parenchyma cells. Calcium concentration in the cytosol increases and activates the jasmonic acid (JA)-mediated response by acting on genes involved in the synthesis and shuttling of lipid intermediates between the chloroplast and the peroxisome. A cell wall receptor may connect with NADPH oxidase triggering a ROS burst in the apoplast. JA, via a negative feedback loop (Denness et al., 2011), controls the levels of ROS and activates the synthesis of flavonoids which act as radical scavengers and in their turn contribute to limit the ROS levels. The MYC2 regulator orchestrates JA response, while TCP transcription factor controls bast fibre elongation by affecting JA levels.

optimal level of JA sustaining elongation.

The above-described model of bast fibre intrusive growth takes into account the recent transcriptomics data and the involvement of ROS. Further studies are necessary to confirm/refute this model. The difficulties of transforming hemp (Andre et al., 2016) is certainly a drawback, however a simple protocol for flax transformation is available (Bastaki and Cullis, 2014) and could provide an important help for functional studies on gelatinous-type bast fibres.

### 2.3. Polar growth

Root hairs and pollen tubes develop in a highly polarized fashion, known as 'polar/tip growth', in which cells elongate unidirectionally and exclusively at the very apex. This specialized growth manner allows cells to rapidly expand and ultimately to produce a tube-like structure with a final length that is several hundred times their original size (Datta et al., 2015; Mangano et al., 2017). In this process, ROS were identified as key players based on the previous studies of NOXs-deficient *Arabidopsis* mutants, where the root hairs failed to elongate due to the suppression of ROS production (Foreman et al., 2003). Further studies have demonstrated that oscillatory levels of ROS are interconnected with $Ca^{2+}$ gradients and pH fluctuations, constituting positive feedback loops to modulate polar growth over time (Braidwood et al., 2014; Mangano et al., 2016; Wolf and Höfte, 2014). During the polar cell growth, apoplastic ROS ($_{apo}$ROS) production is triggered by high level of cytoplasmic $Ca^{2+}$ in the apical zone via reactions mainly catalyzed by NOXs. Subsequently, high concentration of ROS transiently elevate the $Ca^{2+}$ level by activating plasma membrane $Ca^{2+}$ channels, resulting in a tip-high gradient that is required for polar growth (Duan et al., 2014; Foreman et al., 2003). It is likely that pH oscillations can affect NOXs activity via changing the protein conformation and the binding affinity of $Ca^{2+}$ (Mangano et al., 2018). Moreover, Monshausen and colleagues reported that ROS and pH may act in a coordinated and complementary way to regulate cell wall properties thereby sustaining polar growth, although the underlying mechanism remains to be explained (Monshausen et al., 2007).

Homeostasis is essential in alleviating the toxicity of ROS and permitting normal polar cell growth, therefore the level of ROS is under tight regulation (Mangano et al., 2016, 2018). NOXC (Foreman et al., 2003; Monshausen et al., 2007) and NOXH/NOXJ (Boisson-Dernier et al., 2013; Wu et al., 2010) are mainly responsible for $_{apo}$ROS production in growing root hairs and pollen tubes, respectively. On the other hand, ROS-scavenging enzymes, such as CATs, APXs, can restore homeostasis if ROS concentration spikes above optimum (Mangano et al., 2018). Additionally, different forms of ROS have been shown to play distinct roles in polar growth. For example, $OH^{\bullet}$ are associated with the cell wall relaxation process by cleaving cell wall polysaccharides (Fry, 1998), while $H_2O_2$ are involved in the cell wall rigidification by assisting in the formation of covalent linkages between cell wall phenolic compounds and structural proteins (e.g. extensins) (Mangano et al., 2016c; Xiao et al., 2014). It is noteworthy that secreted type-III peroxidases modulate the balance between different ROS and confer a direct effect on $_{apo}$ROS homeostasis through an antagonistic manner: producing ROS in oxidative cycles, whereas metabolizing ROS in hydroxylic and peroxidative cycles (Mangano et al., 2018; Passardi et al., 2004). Further work is still needed to enrich our understanding of ROS regulation in polar growth, for example, to understand the mechanism of activation of $Ca^{2+}$ channels by ROS and in the self-incompatibility response (Wang et al., 2010).

### 2.4. Organ senescence

ROS have been considered as key signaling actors during natural senescence, the final stage in the development of a plant organ (Becker and Apel, 1993). The effect of ROS on plant senescence has been widely studied in leaves and petals. Rogers and colleague concluded that the mechanisms of ROS generation and scavenging are similar in both organs, however photosynthesis-derived ROS, reversibility of senescence, nutrient remobilization are different, mainly because of the unique functionalities of leaves and petals (Rogers and Munné-Bosch, 2016). It has been proposed that senescence is triggered by the accumulation of ROS and, interestingly, different forms of ROS may function in a different manner with their own spatial- temporal pattern.

Among ROS, the roles of $H_2O_2$ have been most thoroughly investigated in this respect as a result of their better stability and capability to diffuse through membranes (Quan et al., 2008; Yang and Poovaiah, 2002). Numerous studies have suggested that $H_2O_2$ can act as signaling molecules to promote senescence in different plant species and participate in a complex regulatory network orchestrating senescence process (Bieker et al., 2012; Jajic et al., 2015). It has been shown that external application of $H_2O_2$, or treatments that trigger the production of $H_2O_2$ can induce the expression of several senescence-regulated NAC transcription factors (Balazadeh et al., 2011; Davletova et al., 2005; Gadjev et al., 2006; Gechev and Hille, 2005). Furthermore, a study on barley has revealed that the production of $H_2O_2$ peaked right after the induction of senescence and at the very end of the senescence process, indicating a dual role of $H_2O_2$ during leaf senescence: one is in the induction of senescence as a signal molecule and another is in the final degradation of the cell structure at the late stages of senescence (Jajic et al., 2015).

In addition to $H_2O_2$, the production of $O_2^{\bullet-}$ was also reported to increase during the process of natural (Pastori and Del Rio, 1997) and artificially-induced senescence (McRae and Thompson, 1983). During the development of barley, $O_2^{\bullet-}$ levels were observed to continuously increase and reach the highest concentration right after the onset of senescence, followed by a constant decline till the end of the senescence process (Jajic et al., 2015). In tobacco, an increase in the $O_2^{\bullet-}$ level was also detected in the interveinal area of senescing leaves and in the minor veins of mature and senescent leaves (Niewiadomska et al., 2009). Taken together, it is reasonable to speculate that the spatial-temporal production of ROS is crucial for the non-uniformed suppression of photosynthesis-related genes during plant senescence (Niewiadomska et al., 2009).

## 3. Toxic concentrations of metals, ROS and impact on plant secondary cell wall

The plant cell wall is a natural composite material made up of a chief load-bearing component, cellulose, interwoven with matrix polysaccharides (pectins and hemicelluloses), as well as proteins and, in some instances, impregnated by the aromatic macromolecule lignin (Guerriero et al., 2015, 2016). A strong body of evidence in the literature has demonstrated a role of the cell wall in the response of plants to toxic concentrations of heavy metals: besides acting as a "reservoir" for the accumulation of heavy metals (via ion exchange mechanism with components of the cell wall containing $-COOH$ groups, notably low methyl-esterified homogalacturonans), the plant cell wall is remodeled at the onset of heavy metal stress (Gall et al., 2015; Krzesłowska, 2011; Parrotta et al., 2015).

In this paragraph, we will discuss the ROS-associated cell wall modification accompanying the response to heavy metal stress, namely lignification in SCWs (Loix et al., 2017). The remodeling of pectins, i.e. an increase in low methyl-esterified (acidic) pectins sequestering heavy metals in the "egg-box" structure, in primary cell walls upon heavy metal stress has already been reviewed by us previously (Parrotta et al., 2015).

One of the promptest effects taking place in the cell wall of plants exposed to an exogenous stress is a burst of ROS generated by NOX (Raggi et al., 2015): the generation of $O_2^{\bullet-}$ leads to the formation of $H_2O_2$ via the action of SOD and in the cell wall class III peroxidases catalyze oxidation of various substrates (via the peroxidative cycle), which results in cell wall cross-linking and growth arrest (Passardi

et al., 2004). Peroxidases, however, can also promote cell wall loosening (and hence favor expansion) via the hydroxylic cycle, during which the Fenton reaction produces $OH^{\bullet}$ that will trigger the non-enzymatic cleavage of cell wall polysaccharides (Schweikert et al., 2002).

Heavy metal stress has been shown to promote lignification in plant SCWs in both herbaceous and woody species. A comparison between thale cress and the metal hyperaccumulator *Thlaspi caerulescens* under Zn treatment showed an upregulation, in *Thlaspi*, of genes related with lignin and suberin biosynthesis: the higher expression of these genes is linked to the U-shaped lignification/suberization of the root endodermis in the hyperaccumulator (Van De Mortel et al., 2008). The deposition of "stress lignins" is indeed a recognized feature and a protective mechanism against e.g. pathogen entry and abiotic stress (Moura et al., 2010). Lignin formation relies on the provision of monolignols, which originate from the phenylpropanoid pathway; the activation of this pathway is often triggered by heavy metal stress (see also next paragraph).

In alfalfa stems exposed to Cd, a proteomic approach identified several peroxidases that were more abundant and their corresponding genes were also upregulated after stress treatment (Gutsch et al., 2018). Soybean seedlings exposed to Cd displayed increased phenylalanine ammonia-lyase (PAL) and peroxidase activity, as well as an increase in H and S lignin units (Finger-Teixeira et al., 2010). In soybean and lupin exposed to 15–25 mg/L Cd or 150–350 mg/L Pb the activity of PAL was induced. However, this increase was not correlated with an induction of gene expression and lignin accumulation in lupin (Pawlak-Sprada et al., 2011). This suggests the presence of a transcriptional and post-transcriptional regulation and the involvement of the activated phenylpropanoid pathway in the synthesis of secondary metabolites other than lignin building blocks.

In the woody species *Populus* x *canescens* the application of $CdSO_4$ resulted in an increase in protective soluble phenolic compounds (He et al., 2011). These compounds were more abundant in the bark with respect to the wood, while, conversely, ROS were produced at a higher level in the wood. These results highlight the presence of a different response to heavy metals in the organs of the same tree, which relates with the higher/lower capacity to synthesize specific secondary metabolites.

## 4. Toxic concentrations of metals, ROS and plant secondary metabolite production

Plants uptake metals via roots and transport them into various plant tissues, where their presence triggers physiological, as well as genetic changes (Ahmad et al., 2016). Toxic concentrations of metals interfere with the plant physiological status by inhibiting germination and growth (Latef, 2018), altering carbohydrate and protein contents (Mondal et al., 2013) and perturbing nutrient uptake (Siddiqui et al., 2012). Some metals (like Cu, Zn, Mn, Ni and Fe) play fundamental roles in plant cells as key components of photosystems and enzymes (Page and Feller, 2015). An excess of metals (and especially heavy metals) can however be detrimental to plants, therefore plant cells put in place mechanisms avoiding their toxic accumulation. In this respect, recent studies focused the attention on secondary metabolite production under heavy metal stress in plants, because of their chelation ability (Eghbaliferiz and Iranshahi, 2016). So far, the scientific interest has mainly been devoted to the contents of functional molecules, such as polyphenols, vitamins and carotenoids that were shown to display a varying trend depending on the plant species and on the exposure to different heavy metals (Asgari Lajayer et al., 2017; Kısa et al., 2016).

Babula and colleagues studied *Hypericum perforatum* plants for their physiological responses to lanthanum and cadmium excess in different tissues, notably shoots and roots (Babula et al., 2015). The results showed a general raise in some phenolic acids (e.g ferulic acid) and, on the contrary, a decrease of flavonoids (e.g epicatechin and procyanidin), both in the shoots and in the roots. Interestingly, another study reported a direct correlation between heavy metal accumulation and the expression of *PAL* (Ma et al., 2016), the first gene intervening in the phenylpropanoid pathway. These data witness the impact of heavy metals on genes involved in the production of phenylpropanoids, thereby providing an explanation for the accumulation of phenolic acids in heavy metal-stressed plant cells.

One possible explanation for a differential impact of metals on the phenylpropanoid pathway is the following. Plants may rather invest in the first steps of the pathway to produce phenolic acids (hydroxycinnamic acids) and do not activate the genes intervening in the subsequent steps (which lead to the formation of flavonoids and anthocyanins) as a way to save energy, while being able to counteract stress via phenolics. As it was previously discussed, phenolic compounds are synthesized in response to heavy metal stress as a protective mechanism: phenolics are powerful Cd chelators and their production is for example enhanced in *Matricaria chamomilla* roots (Kovácik and Klejdus, 2008). The preferential activation of genes involved in the early phenylpropanoid pathway may also coincide with an increased production of monolignols and, ultimately, lignin.

It would be interesting to measure, in future studies, the gene expression changes accompanying the decreased flavonoid content reported by some studies in heavy metals-stressed plants: the targeted analysis of those genes involved in the biosynthesis of flavonoids (e.g 4-coumarate-CoA ligase, chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase; Rigano et al., 2016), as well as of the transcriptional master regulators (e.g *MYB*s; Liu et al., 2015) will provide important clues on the differential regulation of the phenylpropanoid metabolic branches.

Heavy metal concentration is a crucial parameter affecting the response of plants and the impact on secondary metabolism. For example, Ibrahim and colleagues tested different concentrations of cadmium and copper (cadmium 2 mg/L–4 mg/L; copper 70 mg/L–140 mg/L) on *Gynura procumbens* plants and reported that lower levels of heavy metals enhance the production of secondary metabolites; on the contrary, higher combined metal levels inhibit the synthesis of secondary metabolites in plants (Ibrahim et al., 2017).

Exposure time is the second factor that should be considered when evaluating the impact of metals on plant responses (Dupae et al., 2014). In an ongoing study on alfalfa plants exposed to a low concentration of Cd for an entire season a high accumulation of conjugated isoflavones was observed (Gutsch et al. submitted). This intense accumulation of secondary metabolites in plants that, after overcoming a significant phenotypic impact in the first weeks after sowing, are doing well, indicates that secondary metabolism has a prime function in the long-term tolerance of heavy metal exposure.

Within plant cells there is a dynamic balance between oxidants and antioxidants that keep ROS accumulation under control (Lobo et al., 2010). If this physiological equilibrium is unbalanced towards the accumulation of oxidants, the cell responds with the production of compounds able to restore the initial state (Fig. 2). The role of secondary metabolites in counteracting ROS stress is well documented (Bartwal et al., 2013). Polyphenols, terpenes and vitamins are the chief secondary metabolites able to counteract ROS and avoid oxidative stress (Sharma et al., 2012). The chemistry of these molecules is responsible for their scavenging activities: as an example, it is here worth mentioning that the antioxidant property of flavonoids is dictated by their hydroxyl groups which donate hydrogen and an electron to radicals, thereby stabilizing them (Heim et al., 2002).

We believe it is important to discuss also the impact of nanoparticles (NPs, for example metal oxide NPs) on plant secondary metabolism, since they engender ROS (Rastogi et al., 2017). NPs-induced ROS are produced both at the apoplast, via the action of plasma membrane-bound NOX and the chloroplast (Marslin et al., 2017); they activate intracellular signaling cascades (calcium and MAPK), which involve the same players previously described for intrusive growth. Plant hormones play an important role in the response to abiotic stress (such as NPs)

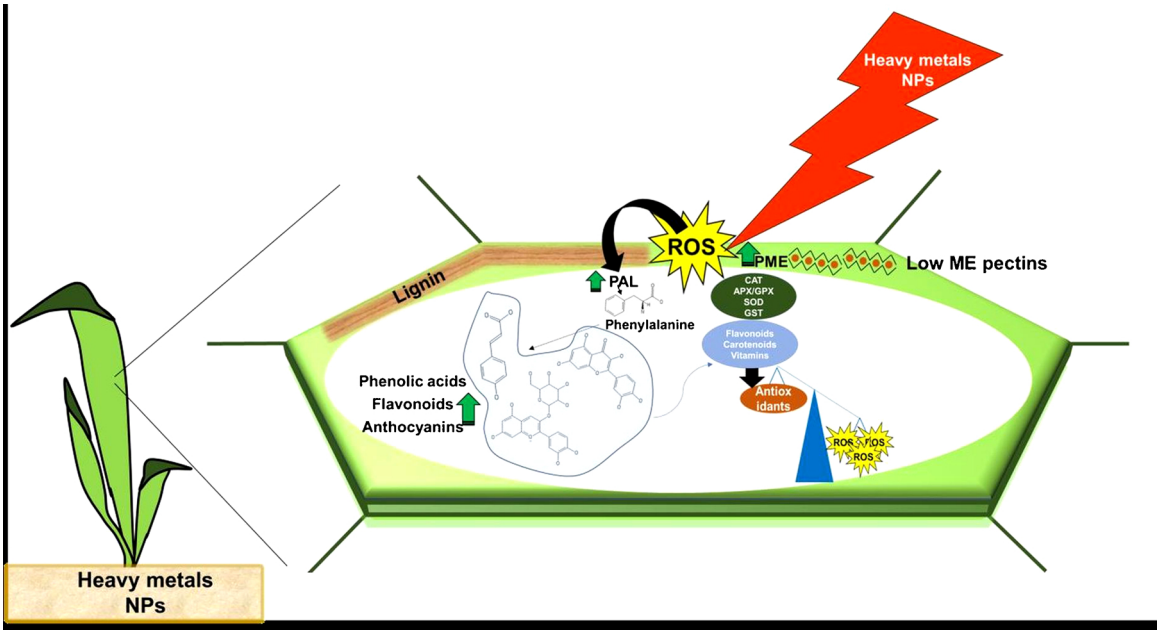


**Fig. 2.** Cartoon depicting the major events accompanying heavy metal and metal oxide nanoparticle (NPs) stress. The stress triggers a ROS burst in the apoplast which exerts an induction on *PAL*. The phenylpropanoid pathway is boosted with the subsequent production of phenolic acids, flavonoids and anthocyanins. These secondary metabolites, together with the enzymatic antioxidant system, contribute to counterbalance ROS. At the cell wall-level an increased lignification is observed, as well as a modification of the pectins (increase in acidic pectins) which can sequester heavy metals via the "egg-box" structure. PME: pectin methylesterase; ME: methyl-esterified; SOD: superoxide dismutase; APX/GPX: ascorbate/guaiacol peroxidase; GST: glutathione-*S*-transferase; CAT: catalase.

and in this perspective JA is a key transducer of the NPs-driven stimulus on plant secondary metabolism. Barley plants exposed to CdO nanoparticles displayed alterations in both primary and secondary metabolism (Večeřová et al., 2016): a significant increase in total amino acids was observed in roots as well as leaves, together with a decrease in saccharides in the roots of exposed plants. Isovitexin increased by more than 180% in the leaves of plants where aboveground biomass only was exposed to Cd NPs.

Hazel cells treated with Ag NPs showed an increased production of taxol and baccatin III, accompanied by a parallel increase in total soluble phenols; interestingly, the produced taxanes were able to kill cancerous HeLa cells (Jamshidi and Ghanati, 2017).

The stimulatory effect of NPs can be exploited to elicit the production of specific secondary metabolites (e.g. those produced in small amounts), or to nanotrap them via adsorption (Marslin et al., 2017). For this last application, green synthesized NPs may offer an important advantage over those manufactured via conventional chemical methods (Marslin et al., 2017). The stimulation of secondary metabolite production by heavy metals and metal oxide NPs could thus be harnessed for non-food crops, e.g. aromatic and medicinal plant, cultivated on contaminated soils and used for essential oil production (Maleki et al., 2017). The process of distillation does not remove heavy metals from the biomass; hence, the final product does not contain detectable amounts of heavy metals (reviewed by (Asgari Lajayer et al., 2017).

To conclude this section on the impact of ROS on plant secondary metabolite production, we will also discuss shortly the role of NO in cell culture elicitation, in the light of its cross-talk with ROS and its physiological impact. Cell culture elicitation with fungal elicitors (e.g. cerebrosides, cell wall extracts) are accompanied by a NO burst which precedes the accumulation of secondary metabolites (saponins, artemisinin, to mention some well-known metabolites). Additionally, mechanical stress also induces NO production: ultrasound was shown to cause NO-dependent accumulation of taxol and baccatin III in *Taxus yunnanensis* (Zhang et al., 2012 and references therein). Notably, an effect of NO on the first committed step of the phenylpropanoid pathway (PAL) was described in *Ginkgo biloba* callus, which was accompanied by an enhanced production of flavonoids (reviewed by Zhang et al., 2012).

## 5. Conclusions and future perspectives

The studies described above show that concentration and exposure time are the crucial parameters determining the impact of heavy metals on plant secondary metabolite production. In this respect, future studies should devote efforts towards determining, experimentally, threshold concentration values able to downregulate or upregulate secondary metabolite production. These threshold values will certainly be species-dependent; the response of the metabolome and of the genes involved in secondary metabolism in hyperaccumulators will likewise be interesting to assess via comparisons with non hyperaccumulators from the same species (for example *Thlaspi* species or the model species *Arabidopsis thaliana* and *A. halleri*).

Comparisons among facultative hyperaccumulators can provide important ecological and evolutionary insights concerning hyperaccumulation (Pollard et al., 2014) and such studies can address whole plants, as well as different tissues and undifferentiated calli (Lefèvre et al., 2010). The use of RNA-Seq can provide a depth of analysis allowing the identification of the molecular mechanisms underlying the improved ROX-detoxifying capacity of hyperaccumulators (Meier et al., 2018).

An interesting and promising line of future research involves also the study of ancient local non-commercial varieties of crops as alternative sources of secondary metabolites with nutraceutical properties and genes conferring (a)biotic stress resistance. Non-commercial varieties of fruits, as for example apples, were indeed shown to possess higher contents of antioxidant capacity even after drying (Francini et al., 2017), in particular polyphenols (phloridzin, chlorogenic acid, epicatechin, catechin) were found in high abundance. Often ancient local varieties show agricultural benefits linked to their lower fertilizer requirement and enhanced resilience against environmental stresses: these plants were indeed not subjected to selection following commercial parameters and thrive in environments with minimal human impact (Berni et al., 2018). Such native ancient species display the best adaptation to the environment and hence can be of scientific interest:

for example, comparative studies addressing the response to exogenous stresses of local varieties and the respective commercial counterparts can shed light on the adaptive mechanisms put in place by the former and on the link with the production of secondary metabolites.

## Author statement

Conceptualization RB, J-FH, GC, GG; funding acquisition J-FH, GG; project administration J-FH, GC, GG; Writing - review & editing all the authors.

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