



## Ameliorative effects of exogenous melatonin to heavy metal and other abiotic stress tolerance in plants

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### Abstract

Heavy Metal (HM) toxicity is defended by plants using mechanisms such as metal chelation and vacuolar sequestration. HM-induced oxidative stress is also limited in plants by routine scavenging of free radicals including reactive oxygen/nitrogen species (ROS/RNS) generation. In this review, we focus on exogenous melatonin treatment to regulate its biosynthetic pathway, the metal accumulation, and redox system. Furthermore, the photosynthetic components are significantly modulated in melatonin-treated plants under metal and other abiotic stressed conditions. Melatonin is also involved in nitric oxide (NO) signaling. Evidence suggests that the improvement in stress tolerance is due to the application of melatonin in a dose-dependent manner. Reports have suggested that phytohormones such as abscisic acid and jasmonic acid are mediated by melatonin. The genes related to secondary metabolites including flavonoid, phenolic, and saponin biosynthesis are mainly over-expressed in presence of melatonin.

**Keywords:** Melatonin, Heavy metals (HMs), Oxidative stress, Phytohormones, Secondary metabolites

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### 1. Introduction

Heavy Metals (HMs) are naturally obtained from weathered metallic rocks and volcanic beds. Anthropological activities through mining, industrial effluents, building construction, and agricultural activities including the use of fertilizers and pesticides have also contributed the HM toxicity. The HMs are determined based on various parameters such as density, atomic number/ mass, chemical properties, and toxicity level (Duffus, 2002). They have an atomic number and density of more than 23 and 5 g cm<sup>-3</sup> respectively (Deblonde *et al.*, 2011). HMs such as copper (Cu), zinc (Zn), chromium (Cr), and nickel (Ni) are essential micronutrients while, lead (Pb), cadmium (Cd), arsenic (As), mercury (Hg) are xenobiotic metals. Due to HM accumulation, plants suffer from stunted growth, reduced biomass, impaired photosynthesis, and chlorophyll degradation. Other

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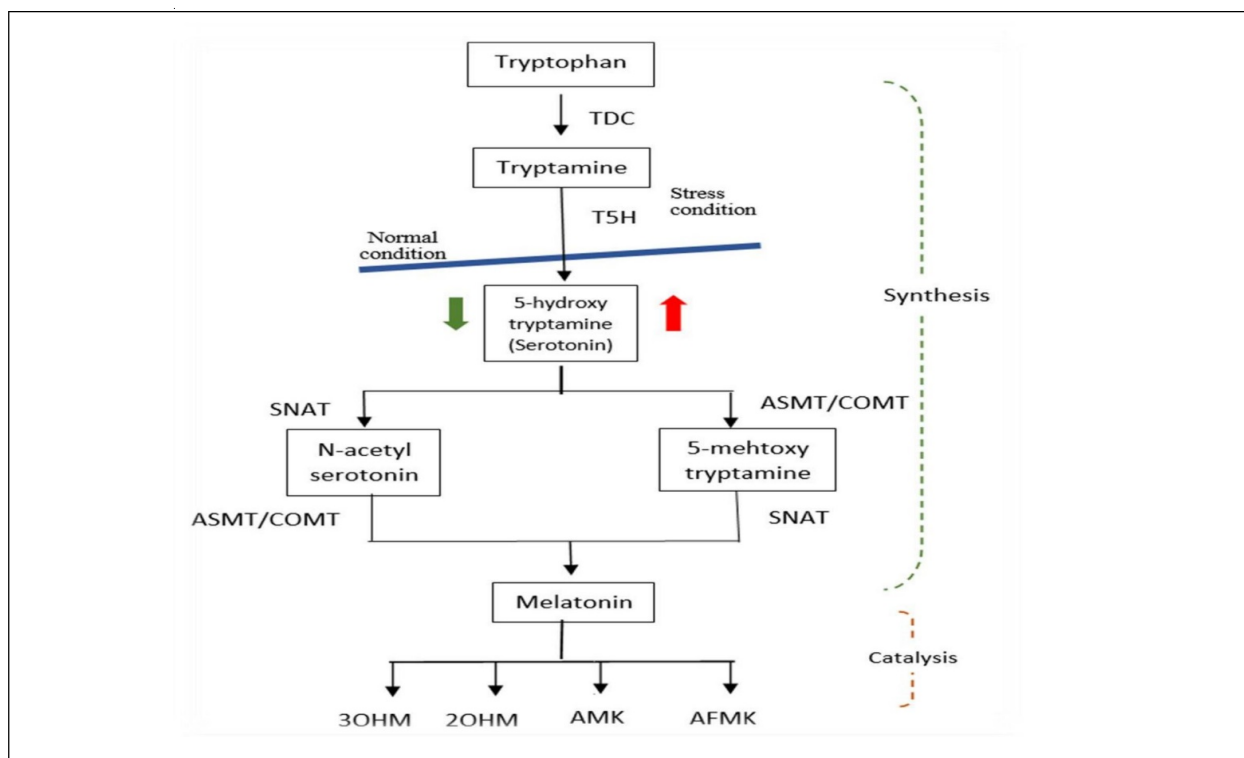
symptoms include abnormal mineral, and water uptake, ion imbalance, and disturbed redox equilibrium (Ackova, 2018; and Nagajyoti *et al.*, 2010). Abiotic stressors such as drought (Ibrahim *et al.*, 2020; Naghizadeh *et al.*, 2019; and Sharma and Zheng, 2019), salinity (Li *et al.*, 2012; Siddiqui *et al.*, 2019; and Zhou *et al.*, 2016), high (Manafi *et al.*, 2021; and Xu *et al.*, 2016) or low (Nawaz *et al.*, 2021) temperature, pH (Arnao *et al.*, 2020), light intensity (Lee and Back, 2021), UV-B radiation (Yao *et al.*, 2021; and Zhao *et al.*, 2012), and herbicides (Park *et al.*, 2013) cause plant damages in a similar fashion.

Melatonin is a ubiquitous and pleiotropic molecule that was discovered in the bovine pineal gland (Lerner *et al.*, 1958). Later on, it was also found in plants, i.e., phytomelatonin by two separate groups of scientists (Dubbels *et al.*, 1995; and Hattori *et al.*, 1995). Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine derivative of tryptophan with a molecular weight of 232.2 g/mol, containing a 3-amide group and 5-methoxy group which serves as ROS quenching sites (Kaur *et al.*, 2015). It can easily pass through the plasma membrane because of its amphiphilic nature and participates in a wide variety of functions ranging from increased seed germination to delaying of leaf senescence (Wang *et al.*, 2012; and Wei *et al.*, 2015). The discovery of the first phyto melatonin receptor (CAND2 PMTR1) is involved in stomatal closure via GPCR mediated signaling and ABA-H<sub>2</sub>O<sub>2</sub> crosstalk (Wei *et al.*, 2018). However, the function of CAND2 PMTR1 is opposed in another report (Lee and Back, 2020). Phytomelatonin is widely studied for protective action against abiotic stressors due to its free radical scavenging activity across different plant families such as Brassicaceae (Menhas *et al.*, 2021), Fabaceae (Elsayed *et al.*, 2020; and Siddiqui *et al.*, 2020), Asteraceae (Namdjoyan *et al.*, 2020), and Solanaceae (Jahan *et al.*, 2020). Previously, phytomelatonin was detected by radioimmunoassay (RIA) (Dubbels *et al.*, 1995) and gas chromatography with mass spectrometry (GC-MS) but now the most applied and recommended techniques for identification and quantification is liquid chromatography and mass spectrometry (LC-MS) (Arnao, 2014). Since its detection in plants, the function of melatonin has been documented in regulating various physiological processes. In this review, we have summarized its interaction with melatonin biosynthesis, metal detoxification, ROS scavenging activities, and crosstalk with other plant metabolites to enhance the defensive action in plants against the HM stress and other abiotic stresses.

## 2. Phytomelatonin biosynthesis and abiotic stress

The biosynthesis of melatonin in plants is illustrated in figure1 (Back *et al.*, 2016; and Hardeland, 2016). In stressed conditions, ASMT/COMT enzyme is employed by a high serotonin level for methylation and 5-methoxy tryptamine is produced; followed by acetylation using SNAT and melatonin is produced (Lee and Back, 2017; Tan and Reiter, 2020; and Ye *et al.*, 2017). Thus, there is no fixed regulation for the final step in melatonin biosynthesis, and enzymes including SNAT, COMT, and melatonin 2-hydroxylase may be equally important as ASMT for determining melatonin contents in plants (Byeon *et al.*, 2015). In plants, COMT activity is alternative to ASMT and both of these enzymes can be found in the cytoplasm whereas SNAT enzyme is localized in chloroplasts, as observed in rice (Byeon *et al.*, 2014). Melatonin can be further catalyzed by melatonin 3-hydroxylase (M3H) (Lee *et al.*, 2016) and melatonin 2-hydroxylase (M2H) (Byeon *et al.*, 2015) into two dominant metabolites of melatonin i.e., cyclic 3-hydroxymelatonin (3-OHM) and 2-OHM cyclic 2-hydroxymelatonin (2-OHM) respectively. M3H and M2H belong to 2-oxoglutarate dependent dioxygenase (2-ODD) family proteins (Bugg, 2003). 3-OHM is widely studied for chelation cascades in Cu ions-induced oxidative stress (Galano *et al.*, 2015). Other metabolites of melatonin such as N1-acetyl-5-methoxykynuramine (AMK), N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) are also investigated in Cu chelation (Galano *et al.*, 2014).

The biosynthesis of melatonin is regulated by different abiotic stressors including HMs. TDC, SNAT, and ASMT activity are induced by high or low-temperature stress and Cu stress in *Vitis vinifera* (Wang *et al.*, 2019). The genes related to melatonin biosynthesis (ASMT1, ASMT2, ASMT3, TDC, and T5H) under saline conditions have been upregulated in peanut (*Arachis hypogaea*) seedlings (Elsayed *et al.*, 2020). Some of the stress-regulating genes including, AREB1, AIM1, MAPK1, WRKY33, and CDPK1, are upregulated by the overexpressed *caffeic acid O-methyl transferase 1* (SICOMT1) of tomato plants under saline stress (Liu *et al.*, 2019). The expression of genes related to melatonin biosynthesis is regulated by melatonin pretreatment in watermelon seedlings under vanadium stress (Nawaz *et al.*, 2018).



**Figure 1: Biosynthetic pathway of melatonin under normal and stressed condition**

**Note:** TDC = Tryptophan Decarboxylase; T5H = Tryptamine 5-Hydroxylase; SNAT = Serotonin N-Acetyl Transferase; ASMT = Acetyl Serotonin Methyl Transferase; COMT = Caffeic Acid O-Methyl Transferase; 3OHM = Cyclic 3-Hydroxymelatonin; 2OHM = Cyclic 2-Hydroxymelatonin; AMK = N1 -Acetyl-5-Methoxykynuramine; AFMK= N1-Acetyl-N2-Formyl-5Methoxykynuramine.

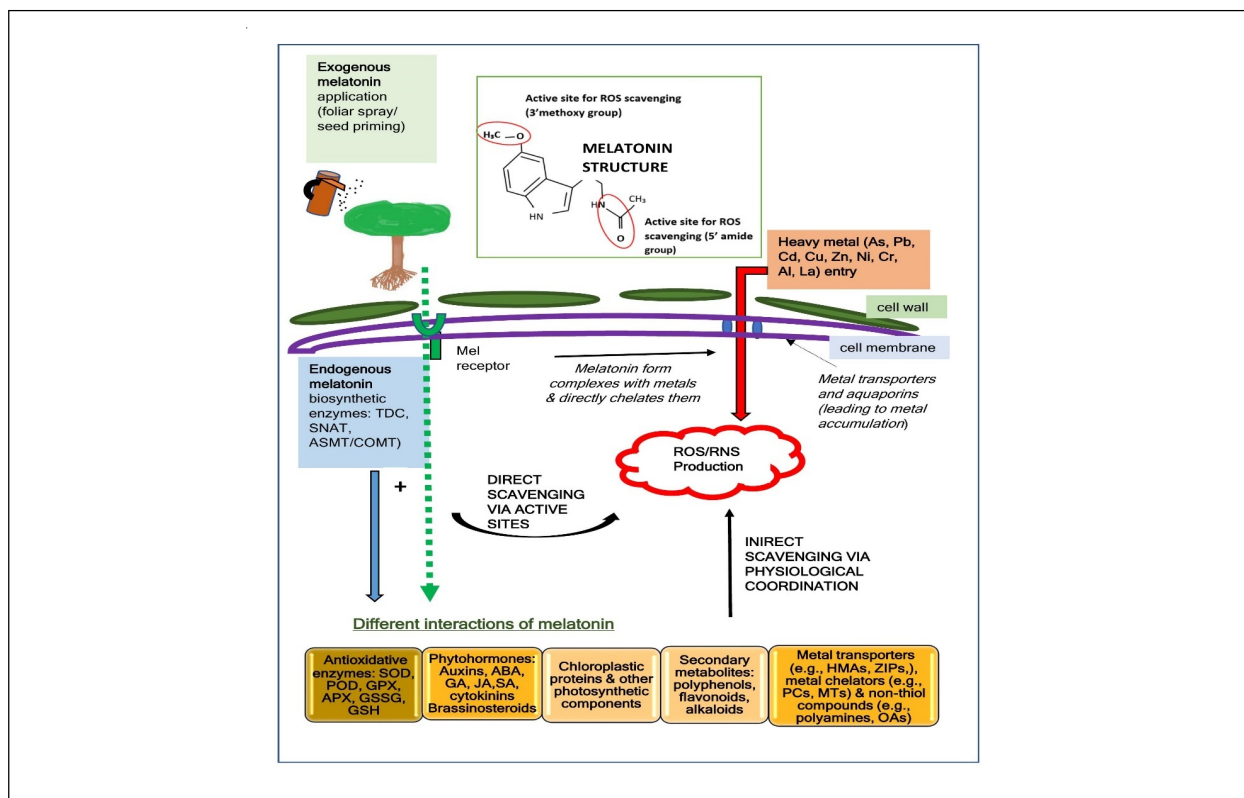
### 3. Melatonin and heavy metal uptake in plants

#### 3.1. Melatonin aids metal translocation and accumulation in plants

Metal-ligand complexes get distributed to apoplast tissues (trichomes and cell walls) followed by storage in vacuoles. It is reported that HMs (Zn, Pb, Co, Cd, Ni, and Cu) are mostly localized in the cell walls of the root region (Sousa *et al.*, 2008). Cell wall trapping is an important route for copper detoxification and the expressions of genes related to the cell wall biosynthesis such as *CESA* (cellulose synthase A), *CSL* (cellulose synthase-like), *PME* (pectin methyltransferase), and *XTH* (xyloglucan endotransglucosylase/hydrolase) are enhanced by melatonin in cucumber (Cao *et al.*, 2019). The expressions of cell-wall remodeling-related genes *LBD16* (lateral organ boundaries domain) and *XTR6* (xyloglucan endotransglycosylase-related) are induced by both melatonin and serotonin in the local milieu which promotes lateral root development (Wan *et al.*, 2018). It is confirmed that the role of exogenous melatonin in metal accumulation is largely dose-dependent. It has been observed that 150  $\mu\text{M}$  melatonin is optimum for Cd accumulation in both root and shoot regions of *Perilla frutescens* whereas treating with 200  $\mu\text{M}$  melatonin increases significant Cd concentration in only roots (Xiang *et al.*, 2019). Increased thickness in the root epidermal and cuticular region has been observed when treated with 400  $\mu\text{M}$  melatonin which is beneficial for limiting the Pb translocation into the shoots of *Amaranthus cruentus*. The cuticular thickness also protects from dehydration and provides physical support to the disintegrated root cells (Jolayemi, 2020). The most common metal transporters in plants include HM ATPases [HMAs] (Chao *et al.*, 2012; and Takahashi *et al.*, 2012), ATP binding cassette [ABC] transporters (Zhang *et al.*, 2018), natural resistance-associated macrophage protein [NRAMP] (Milner *et al.*, 2014; and Sasaki *et al.*, 2012), zinc-regulated and iron-regulated transporter-like protein [Zrt, Irt-like, ZIP family] (Lin *et al.*, 2009), and yellow stripe 1-like protein [YSL] (Curie *et al.*, 2009) are all responsible for metal uptake and accumulation. A crucial role has been played in metal sequestration by melatonin alone or in combination with other molecules by modulating these metal transporters genes and using them as ligands for transportation (Table 1, Figure 2). The metal transporter genes that belong to *ZIP*, *YSL*, and *ABC* gene families are altered by melatonin in presence of Cu toxicity (Cao

<b>Table 1: Metal transporters/chelators and thiol/non-thiol compounds that are regulated by melatonin treatment along with their concentration in respective plant species under different HM doses</b>						
S. No.	Heavy metals (dose)	Plant Species	Melatonin concentration	Modulated metal transporters/chelators related genes and thiol/non-thiol compounds	Upregulation (↑) Downregulation (↓)	References
1.	Cadmium (25 and 100 mM)	<i>Solanum lycopersicum</i> (Tomato)	25, 50, 100, 250, and 500 $\mu$ M	GSH, PC <sub>2</sub> , PC <sub>3</sub> , and PC <sub>4</sub>	↑	(Hasan <i>et al.</i> , 2015)
2.	Cadmium (50 $\mu$ M)	<i>Raphanus sativus</i> L. (Radish)	10, 25, 50, 100, and 200 mM	MT1, HMA, ZIP, YSL	↑	(Xu <i>et al.</i> , 2020)
3.	Arsenic (25 $\mu$ M)	<i>Oryza sativa</i> (Rice)	0.25 $\mu$ M	PC, GSH	↑	(Nazarian and Ghanati, 2020)
4.	Cadmium (50, 100, and 200 $\mu$ M)	<i>Medicago sativa</i> (Alfalfa)	50 $\mu$ M	ABC, PDR8, HMA4, NRAMP6	↑ ↓	(Gu <i>et al.</i> , 2017)
5.	Selenium (50, 100, 200 mM)	<i>Brassica napus</i> (Rapeseed)	50, 100 $\mu$ M	Proline, GSH, GSSG, GSH/GSSG, PC, PCS	↑	(Ulhassan <i>et al.</i> , 2019)
6.	Cadmium (20 $\mu$ M)	<i>Brassica campestris</i> spp. chinensis L. (Chinese cabbage)	100 $\mu$ M	IRT1	↑	(Wang and Chen, 2020)
7.	Aluminium (50 $\mu$ M)	<i>Glycine max</i> L. (Soyabean)	0.1, 1 $\mu$ M	citrate, malate	↑	(Zhang <i>et al.</i> , 2017)
8.	Arsenic (150 $\mu$ M)	<i>Oryza sativa</i> (Rice)	20 $\mu$ M	PDH, IDH, SDH, MDH	↑	(Samanta <i>et al.</i> , 2020)
9.	Cadmium (100 $\mu$ M)	<i>Oryza sativa</i> (Rice)	200 $\mu$ M	OsHMA2, OsHMA3, OsIRT1, OsIRT2, OsNramp1, OsNramp 5	↑	(Jiang <i>et al.</i> , 2021)
10.	Lead (200 $\mu$ M)	<i>Raphanus sativus</i> L. (Radish)	50 $\mu$ M	RsABC5, RsYSL7 & RsMT	↑	(Tang <i>et al.</i> , 2021)

**Note:** GSH = Glutathione; GSSG = Glutathione Reductase; PC = Phytochelatin; PCS = Phytochelatin Synthase; MT = Metallothionein; HMA = Heavy Metal ATPases; ABC = ATP Binding Cassette; ZIP = Zinc-Regulated Transporter-like protein ;YSL = Yellow Stripe 1-Like protein; NRAMP = Natural Resistance-Associated Macrophage Protein; PDR = Pleiotropic Drug Resistance; IRT = Iron-Regulated Transporter-like protein ; PDH = Pyruvate Dehydrogenase; IDH = Isocitrate Dehydrogenase ; SDH = Succinate Dehydrogenase; MDH = Malondialdehyde



**Figure 2: Diagrammatic representation of the melatonin-induced regulations in plant cells under HM stress**

**Note:** TDC = Tryptophan Decarboxylase; SNAT = Serotonin N-Acetyl Transferase; ASMT = Acetyl Serotonin Methyl Transferase; COMT = Caffeic Acid O-Methyl Transferase; SOD = Superoxide Dismutase; POD = Peroxidase; GPX = Glutathione Peroxidase; APX = Ascorbate Peroxidase; GSSG = Glutathione Reductase; GSH = Glutathione; ABA = Abscisic Acid; GA = Gibberellic Acid; JA = Jasmonic Acid; HMA = Heavy Metal ATPases; ZIP = Zinc-Regulated Transporter-like protein; PC = Phytochelatin; MT = Metallothionein

*et al.*, 2019). Similarly, the expression of *IRT1* is downregulated by melatonin under Cd (Wang *et al.*, 2021) and Fe (Ahmed *et al.*, 2020) toxicity. The metal transporter genes (*IRT1*, *Nramp1*, *HMA2*, *HMA4*, and *HMA3*) are altered by melatonin in *Nicotiana tabacum* L., which might be involved in reducing Cd accumulation and movement through xylem tissues. Moreover, melatonin could be responsible for limiting Cd uptake and intensifying Cd sequestration into the root vacuoles (Wang *et al.*, 2019). The expressions of *NRAMP1*, *NRAMP5*, *HMA2*, *HMA3*, *IRT1*, and *IRT2* are significantly decreased by the co-treatment of melatonin and silicon in the root regions of *O. sativa*, which has reduced the Cd and As accumulation (Bao *et al.*, 2021). Similarly, *PDR8* (pleiotropic drug resistance 8), *HMA4*, and *NRAMP6* are regulated by melatonin in alfalfa (Gu *et al.*, 2017) and *HMA4*, *ZIP12*, *YSL2*, and *YSL7* are modulated in radish root cells under Cd toxicity (Xu *et al.*, 2020). In a protein docking method, it has been observed that *ZIP8* and *DMT1* (divalent metal transporter 1) form complexes with melatonin and its metabolites. Further, it is reported that the *ZIP8* channel might be involved in Pb transport (Villaseñor-granados *et al.*, 2019). Under Cd stress, gold nanoparticles are used for melatonin uptake and distribution. Here, it has been found that the melatonin content is increased by 18% in roots and 20.3% in leaves whereas Cd content is decreased by 33% and 46.2% respectively. Also, the metal transporter gene expressions, including *OsHMA2*, *OsHMA3*, *OsIRT1*, *OsIRT2*, *OsNramp1*, *OsNramp5*, and *OsLCT1* has been reduced by melatonin-gold nanoparticle treatment in rice roots (Jiang *et al.*, 2021). DNA methylation is an epigenetic modification which is induced by environmental stresses. A declining trend of methylation is observed in HM transporter genes (*RsABCF5*, *RsYSL7*, and *RsHMT*); antioxidant genes (e.g., *RsAPX2*, *RsPOD52*, and *RsGST*) and transcription factors (e.g., *RsWRKY41* and *RsMYB2*) under melatonin influenced Pb stress in radish plants (Tang *et al.*, 2021). The beneficial roles of melatonin in detoxifying HMs including metal sequestration particularly in the root region by these metal transporter families have been indicated in these findings.



### 3.2. Melatonin promotes heavy metal chelation using metal-binding ligands in plants

#### 3.2.1. Thiol compounds (GSH, PCs, and MTs)

A thiol compound consists of a sulfur hydryl (-SH) functional group linked to the carbon atom of alkyl/aryl moiety. Glutathiones (GSH) are tripeptides [ $\{\{\gamma\text{-Glu-Cys}\}_n\text{-Gly}\}$ ] and are the most abundant thiol compounds present in cell organelles (Anjum *et al.*, 2015). Being an intermediate in the ascorbate-glutathione pathway, metals are detoxified by GSH by acting as a precursor for phytochelatin (PC) biosynthesis. PC-metal complexes have been formed by the thiol group derived from the cysteine in the structure. However, with increased HM concentration, PCs get polymerized and stable (metal-PC) $_n$  peptides are produced in different plants (Pal and Rai, 2010; and Clemens and Pers, 2009). The phytochelatin transporters, AtABCC1 and AtABCC2 are have been studied under Cd and Hg stress in *A. thaliana* (Park *et al.*, 2012). As-PC complex is mediated by OsABCC1 and OsPCS1 in rice and further confirms its role in reducing As content (Hayashi *et al.*, 2017). Metallothioneins (MTs) are another low molecular weight, cysteine-rich metal-binding peptide in alleviating HM-induced stress in plants. The role of MTs is defined for balancing essential metals and detoxifying non-essential metals. Pb resistant *P. aeruginosa* strain WI-1 expresses metallothionein by *bmtA* gene is reportedly used as a bioinoculant for pea plants to ameliorate the adverse effects of Pb stress and reduce its uptake into roots and shoots (Naik *et*

**Table 2: Oxidative stress related genes/ enzymes that is modulated by exogenous melatonin under HM stress**

S. No.	Heavy metals (doses in $\mu\text{M}$ )	Plant Species (Common name)	Melatonin concentration	Modulated genes/ enzymes and molecules related to oxidative stress	Upregulation ( $\uparrow$ ) Downregulation ( $\downarrow$ )	References
1.	Cadmium (10 $\mu\text{g/ml}$ )	<i>Cyphomandra betacea</i> (Tamarillo)	50, 100, 150, 200	SOD, POD, CAT	$\uparrow$	(Lin <i>et al.</i> , 2018)
2.	Cadmium (1 $\mu\text{M}$ )	Strawberry ( <i>Fragaria <math>\times</math> ananassa</i> Duch)	10, 50, 100, 150, and 200 $\mu\text{M}$	SOD, CAT, POD, APX_____	$\uparrow$	(Wu <i>et al.</i> , 2020)
3.	Cadmium (25 $\mu\text{M}$ ) & Aluminium (25 $\mu\text{M}$ )	<i>Brassica napus</i> L. (Rapeseed)	50 $\mu\text{M}$ , 100 $\mu\text{M}$	SOD, APX, POD, CAT, GR, GSSG-R	$\uparrow$	(Sami, 2020)
4.	Lead (100 $\mu\text{M}$ )	<i>Zea mays</i> (Maize)	50, 100 $\mu\text{M}$	$\text{H}_2\text{O}_2$ , MDA	$\uparrow$	(Okant and Kaya, 2019)
5.	Lead (50 $\mu\text{M}$ )	<i>Cathamus tinctorius</i> L. (Safflower)	100, 150, 200, 300 $\mu\text{M}$	SOD, CAT, APX, GPX, Gly I, Gly II	$\uparrow$	(Namdjoyan <i>et al.</i> , 2020)
6.	Lead (1200 ppm)	<i>Trigonella foenum graecum</i> L. (Fenugreek)	50 $\mu\text{M}$	SOD, CAT MDA, $\text{H}_2\text{O}_2$ , LOX	$\uparrow$ $\downarrow$	(Xalxo and Keshavkant, 2019)
7.	Lead (800 $\mu\text{M}$ )	<i>Medicago truncatula</i> (Barrelclover)	10 $\mu\text{M}$	SOD, CAT, APX, MDA	$\uparrow$ $\downarrow$	(Zhang <i>et al.</i> , 2020)

Table 2 (Cont.)						
S. No.	Heavy metals (doses in mM)	Plant Species (Common name)	Melatonin concentration	Modulated genes/ enzymes and molecules related to oxidative stress	Upregulation (↑) Downregulation (↓)	References
8.	lead (200 $\mu$ M)	<i>Raphanus sativus</i> L. (Radish)	50 $\mu$ M	<i>RsAPX2</i> , <i>RsPOD52</i> and <i>RsGST</i>	↑	(Tang <i>et al.</i> , 2021)
9.	Arsenic (25 $\mu$ M)	<i>Rosmarinus officinalis</i> L. (Rosemary)	25, 50 $\mu$ M	SOD, POD, CAT, APX	↑	(Farouk and Al-amri, 2019)
10.	Arsenic (5 $\mu$ M)	<i>Vicia faba</i> (Broad bean)	50 $\mu$ M	APX, GR, MDAR, GST, SOD, RuBisco, NADPH oxidase Chlase, $\delta$ -ALAD, GOX	↑ ↓	(Siddiqui <i>et al.</i> , 2020)
11.	Arsenic (25 $\mu$ M)	<i>Camellia sinensis</i> L. (Tea)	100 $\mu$ M	POD, CAT, APX	↑	(Li <i>et al.</i> , 2021)
12.	Copper (80 $\mu$ M)	<i>Cucumis sativus</i> (Cucumber)	0.01 $\mu$ M	SOD, POD and APX	↑	(Cao <i>et al.</i> , 2019)
13.	Copper (10, 25, 50, 75, 100 $\mu$ M)	<i>Brassica napus</i> L., cv. Westar (Canola)	0.1, 1, 10, 100 $\mu$ M	MDA, LPO	↓	(Kholodova <i>et al.</i> , 2018)
14.	Copper (300 $\mu$ M)	<i>Cucumis melo</i> L. (Melon)	10, 50, 100, 300, 500, or 800 $\mu$ M	Redox related genes, LOX genes	↓	(Hu <i>et al.</i> , 2020)
15.	Nickel (50 $\mu$ M)	<i>Solanum lycopersicum</i> (Tomato)	100 $\mu$ M	SOD, CAT, APX, GR, GST, MDHAR, DHAR	↑	(Jahan <i>et al.</i> , 2020)
16.	Iron (high/low supply)	<i>Cucumis sativus</i> (Cucumber)	100 $\mu$ M	Fe-SOD, POD, CAT	↑	(Ahammed <i>et al.</i> , 2020)
17.	Chromium (50, 100 and 200 $\mu$ M)	<i>Zea mays</i> L. (Maize)	0.5, 1.0 $\mu$ M	SOD, POD, CAT and APX	↑	(Malik <i>et al.</i> , 2021)
18.	Aluminium (50 $\mu$ M)	<i>Glycine max</i> L. (Soyabean)	0.1, 1 $\mu$ M	SOD, POD, APX	↑	(Zhang <i>et al.</i> , 2017)
19.	Lanthanum (150 $\mu$ M)	<i>Solanum lycopersicum</i> (Tomato)	150 $\mu$ M	SOD, APX, GR, MDHAR, DHAR	↑	(Siddiqui, Alamri, Alsubaie, <i>et al.</i> , 2019)
20.	Vanadium (50 $\mu$ M)	<i>Citrullus lanatus</i> (Watermelon)	0.1 $\mu$ M	SOD, CAT $H_2O_2$ , MDA	↑	(Nawaz <i>et al.</i> , 2018)
21.	Selenium (50 $\mu$ M, 100 $\mu$ M, and 200 $\mu$ M)	<i>Brassica napus</i> (Rapeseed)	50 $\mu$ M, and 100 $\mu$ M	SOD, APX, GR, and CAT,	↑	(Ul Hassan <i>et al.</i> , 2019)

**Note:** SOD = Superoxide Dismutase; POD = Peroxidase; CAT = Catalase; APX =Ascorbate Peroxidase; GR = Glutathione Reductase;  $H_2O_2$  = Hydrogen Peroxidase; MDA = Malondialdehyde; GPX = Glutathione Peroxidase; GLY = Glyoxygenase; LOX = Lysyl Oxidase; GST = Glutathione S-Transferase; MDHAR = Mono Dehydro Ascorbate Reductase; DHAR = Dehydro Ascorbate Reductase; CHLASE = Chlorophyllase;  $\delta$ -ALAD = Delta-Aminolevulinic Acid Dehydratase; GOX = Glucose Oxidase; LPO = Lipoxygenase

al., 2012). MT proteins have been widely identified and mapped in *Arabidopsis* and *Brassica* species. Among 16 *B. napus* MTs, *BnaMT3C* is shown to be important for As<sup>3+</sup> tolerance (Pan et al., 2018). The genes encoded by *MT 1*, *MT 2a*, *MT 2b*, *MT 3*, and phytochelatin synthase (*PCS*) 1,2 are stated as 35.5%, 28.5%, 4%, 11.3%, 18.7%, and 2% respectively of the total genes that have been identified in *B. rapa* against Cd and Zn tolerance (Liu et al., 2021). Besides Brassicaceae, *Plantago ovata* MTs [MT1, MT2, MT3, MT4] under the Plantaginaceae family have also been identified and characterized (Moulick et al., 2013). The expressions of the *PoMT2* gene and zinc treatment are positively correlated in *P. ovata* (Pramanick et al., 2017). *OsMT2c* gene is also reported to be strongly expressed in rice seedlings (Liu et al., 2015). The metals are indirectly detoxified by melatonin through interaction with GSH, PC, and MT (Table 1, Figure 2). It is reported that the exogenous melatonin enhances the GSH, PCs activity under Cd stress and further improves its accumulation in tomato roots (Hasan et al., 2015). Cd is alleviated in tobacco plants by a melatonin-driven upregulation of *RsMT1* expression level (Xu et al., 2020). More evidence is required to understand the interaction between melatonin and these thiol compounds.

### 3.2.2. Non-Thiol compounds (Organic acid and amino acids)

Organic acids (pyruvic acid, citric acid, oxalic acid, and malic acid) are important intermediates in the Krebs cycle for energy metabolism. These are also multidentate ligands that are involved in HM/metalloid detoxification (Wang et al., 2012). The OA-metal complexes are formed by organic acid anions (OAAs) e.g., malate, citrate, and oxalate; and HM cations found in the soil. Such complexes are then translocated from roots through xylem tissues and get sequestered into the leaf vacuoles. The phytoavailability of trace elements to plants is also increased by OAAs (Han and Lee, 2005). The malate and citrate exudation are enhanced by melatonin under Al toxicity and its concentration in the root region also increased probably due to the upregulated gene encoding acetyltransferase NSI-like (Zhang et al., 2017). The genes related to polyamine biosynthesis, including S-adenosylmethionine decarboxylase (SAMDC) and spermidine synthase (SPDS) are upregulated by melatonin treatment; and hence the polyamine accumulation is observed under heat stress (Gong et al., 2017). Putrescine (Put) and spermidine (Spd) contents are also increased and spermine (Spm) is balanced by melatonin-induced alteration in polyamine metabolism under chilling stress in cucumber (Zhao et al., 2017). Some of the examples of non-thiol compounds modulated by melatonin under HM stress are listed in Table 1.

## 4. Melatonin alleviates oxidative stress in plants

### 4.1. Melatonin scavenges ROS/RNS and stimulates some antioxidative enzymes in plants

Cellular damages are often linked with redox imbalance with impaired regulation, which is more related to reactive and toxic secondary species in the form of reactive oxygen species (ROS). ROS exists in two states in plants: [1] ionic states including hydroxyl radicals ( $\cdot\text{OH}$ ) and Superoxide anions and [2] molecular states including hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and singlet oxygen ( $^1\text{O}$ ) (Blokhina et al., 2003; Apel and Hirt, 2004; Mittler et al., 2004; and Mehta et al., 2021). Superoxide anion is highly unstable, causing redox reactions and thus acts as a precursor for different ROS.  $\text{H}_2\text{O}_2$  is stable inside cells, easily transported through aquaporin channels, and eventually leads to oxidative stress in other parts of the plants (Bienert et al., 2007). Earlier reports have confirmed that melatonin is a potent free radical scavenger and antioxidant (Zhang and Zhang, 2014; and Manchester and Andersen, 2015). Reactive oxygen/nitrogen species (ROS/RNS) are directly and indirectly detoxified by melatonin via regulating the antioxidative and pro-oxidative enzymes. The hydroxyl radical formation during Fenton /Harber Weiss reactions is reduced by melatonin by chelating transition metals (Reiter et al., 2016). The antioxidative protection is also augmented by melatonin in the vicinity of the electron transport chain by improving the reduced GSH contents, and by modulating the glutathione peroxidase (GPX) and Mn-SOD in the mitochondrial matrix along with Cu, Zn-SOD in intermembrane space (Hardeland, 2017). Several melatonin metabolites such as AMK, AFMK, 2-OHM, 3-OHM, 4-OHM, 6-OHM are produced as a result of reactions between different oxidants, primarily ROS and RNS, and these metabolites also act as antioxidants. 3-OHM is much more efficient for peroxy radical scavenging activity, compared to melatonin (Galano et al., 2014). These metabolites are effective for copper chelation and inhibition of Cu<sup>2+</sup>-ascorbate mediated oxidative stress (Galano et al., 2015). The lateral root formation is triggered in both *Medicago sativa* and *Arabidopsis* sp. by regulating  $\text{H}_2\text{O}_2$  which acts as a downstream signaling molecule (Chen et al., 2018).  $\text{H}_2\text{O}_2$  is induced by Cu which stimulates antioxidant enzymes SOD, APX, and CAT via ZmMPK3 (a type of MAPKs) signaling in maize leaves (Jianxia Liu et al., 2018). Some of the important kinases including mitogen-activated



protein kinase (MAPK) and calcium signaling kinases (calcium-dependent protein kinases {CDPKs}, calcineurin B-like interacting protein kinases {CIPKs}, and calcium-related protein kinases {CRKs}) are transcriptionally regulated by exogenous melatonin (Shi *et al.*, 2015). High levels of ascorbic acid (AsA) and glutathione (GSH) and low levels of dehydroascorbate (DHA) and oxidized glutathione (GSSG) is maintained by melatonin-mediated ASA-GSH cycle (Wang *et al.*, 2012). The enzymes such as APX, MDHAR, DHAR, and GR are upregulated by melatonin (Khan *et al.*, 2020). Melatonin along with its metabolites are potential antioxidants against Cu-ascorbate mixtures that are formed during Cu chelation. Enzymes from ascorbate metabolism such as VTC4 and APX 4 are over-expressed by exogenous melatonin under salt stress (Wei *et al.*, 2015). A lot of evidence within the plant kingdom have confirmed that melatonin promotes oxidative stress tolerance under a wide range of abiotic stresses including, drought in maize (Huang *et al.*, 2019), salinity (Siddiqui *et al.*, 2019; and Wei *et al.*, 2015), and HM toxicity (Gu *et al.*, 2017; and Gao *et al.*, 2020). The antioxidative process is triggered by melatonin to combat Pb-stress in safflower plants (Namdjoyan *et al.*, 2020). The combined effects of melatonin and Ca<sup>2+</sup> in broad beans against As toxicity promotes enzymes related to an antioxidant defense mechanism, and influence the ascorbate-glutathione cycle. Several antioxidant enzymes such as SOD, CAT, APX, POD are upregulated in presence of melatonin under HM stress (Table 2, Figure 2).

#### **4.2. Melatonin protects photosynthetic components from ROS damage**

In *Amaranthus tricolor*, the growth is inhibited under copper toxicity, accompanied by reduced net photosynthetic activity and stomatal conductance. A significant decrease in CAT and APX activities, AsA-GSH content, and carotenoid content has been found (KE and Sheng, 2007). Singlet oxygen (<sup>1</sup>O) is usually produced in photosystem II and superoxide anions are produced in chloroplastic electron transport chains (ETCs), mitochondrial ETCs, and membrane dependent NADPH oxidase systems (Bose *et al.*, 2014; and Mhamdi and Van Breusegem, 2018). The photosynthetic rate has been enhanced by melatonin in PSII by improvement in photosynthetic electron transport chain (PET) and D1 protein synthesis under salt stress. The expression of chloroplast genes such as *TRF* and *TRXm* which might be responsible for repairing light reaction components are also influenced by melatonin (Zhou *et al.*, 2016). Similarly, D1 protein degradation is reduced in the presence of melatonin under drought stress in maize seedlings (Huang *et al.*, 2019). It is also reported that melatonin content has increased in high light intensity and salinity stress in the chloroplast region. The relative chlorophyll (chl) and photosynthetic assimilation are also enhanced by melatonin under vanadium stress, along with the reduction in H<sub>2</sub>O<sub>2</sub> and MDA content, and promotion of antioxidant-related genes (Nawaz *et al.*, 2018). The photosynthetic capability concerning chl content and chl biosynthetic genes (protochlorophyllide oxidoreductase, Chlorophyll a oxygenase, and Chlorophyll synthase gene), gas exchange components, and PSII efficiency are enhanced by melatonin (Table 2), while reducing excess Ni assimilation in tomato seedlings (Jahan *et al.*, 2020). Two of the main protein levels of chloroplast protein quality control (CPQC), i.e., caseinolytic protease (Clp) and chloroplast heat shock proteins (CpHSPs) are suppressed along with light-harvesting complex protein levels (Lhcb1, Lhcb4, and RBCL) in the *SNAT1* mutant *Arabidopsis* lines. It is also believed that the melatonin-induced CPQC is partly driven by the MAPK (mitogen-activated protein kinase) signaling pathway (Lee and Back, 2021). MAPK and calcium-dependent signaling pathways might be triggered in presence of Pb-induced stress along with melatonin treatment. Further, it is involved in antioxidative mechanisms and the HM-related genes and TFs (Tang *et al.*, 2021). Moreover, the apoptotic cell death has been avoided by melatonin in tobacco cells (*Nicotiana tabacum* L. line Bright Yellow 2 {BY-2} suspension cells) under Pb stress by preventing the cytochrome c translocation from mitochondria into the cytoplasm (Kobylińska *et al.*, 2017). The apoptotic features such as nuclear fragmentation and condensation, DNA injury, and ROS generation is suppressed by a combination of Ca<sup>2+</sup> ions and melatonin treatment in the stomatal guard cells.

### **5. Melatonin regulates phytohormones and secondary metabolites**

#### **5.1. Crosstalk between melatonin and other phytohormones**

The regulation of phytohormones including abscisic acid (ABA), ethylene, jasmonic acid (JA), in HM mediated signaling have been documented in several studies (Pál *et al.*, 2013; Chmielowska-Bak *et al.*, 2014; and Yan *et al.*, 2015). HM-induced oxidative stress could be also alleviated by these phytohormones (Figure 2). The expressions of genes related to the glutathione synthesis (enzymes including g-glutamylcysteine synthetase, glutathione synthetase, and glutathione reductase) are promoted by JA in Cu and Cd treated *Arabidopsis* plants (Xiang and Oliver, 1998). The cotreatment of JA and Pb in *W. arrhiza* have been shown to inhibit lipid

peroxidation by lowering MDA content (Piotrowska *et al.*, 2009). In another study, oxidative damage is mitigated by JA-treated plants under Pb stress by reducing the expression of transporter genes such as rubredoxin (*RBO*) and P-type ATPase (Bali *et al.*, 2019). Major hormones including ABA, ethanol (ET), salicylic acid (SA), and JA along with the signaling molecules such as kinases, and molecules linked to calcium-mediated signals are believed to be upregulated by melatonin (Table 3). However, genes related to auxin have been down-regulated (Weeda *et al.*, 2014). Seed germination is inhibited while the closing of stomata is promoted by the interaction between melatonin and ABA (Wei *et al.*, 2018). Although, the seed germination is stimulated by melatonin in a mechanism involving CAX3-mediated  $Ca^{2+}$  efflux and  $H_2O_2$  accumulation via ABA degradation and GA synthesis (Li *et al.*, 2021). During heat stress, it has been shown that the endogenous melatonin and gibberellic acid contents are elevated by exogenous melatonin but the ABA content is reduced. Also, GA signaling suppressor and catabolic genes are inhibited while that of ABA catabolic genes are over-expressed with melatonin treatment (Jahan *et al.*, 2021). The knockout mutant *SNAT 2* has been responsible for the flowering

**Table 3: Phytohormones/secondary metabolites along with genes/enzymes related to them that are modulated by exogenous melatonin under HM stress**

S. No.	Heavy metals	Plant species	Melatonin concentration	Phytohormones/ Secondary metabolites	Upregulation (↑) Downregulation (↓)	References
1.	Cu (300 $\mu$ M)	<i>Cucumis melo</i> L. (Melon)	100 $\mu$ M	Jasmonic acid	↓	(Hu <i>et al.</i> , 2020)
2.	Arsenic (75 mg/kg air-dried soil)	<i>Rosmarinus officinalis</i> L. (Rosemary)	25 $\mu$ M, 50 $\mu$ M	Phenolics, flavonoids (anthocyanins)	↑	(Farouk and Al-amri, 2019)
3.	Copper (100 $\mu$ M)	<i>Nicotiana tabacum</i> L. (Tobacco)	100 $\mu$ M	Phenolics, flavonoids	↑	(Wang <i>et al.</i> , 2019)
4.	Fe	<i>Cucumis sativus</i> L. (cucumber)	100 $\mu$ M	PAL, PPO activity	↑	(Ahmed <i>et al.</i> , 2020)
5.	Ni (50 $\mu$ M)	<i>Solanum lycopersicum</i> L. (Tomato)	100 $\mu$ M	Phenols, flavonoids, anthocyanin; PAL, CHS	↑	(Jahan <i>et al.</i> , 2020)
6.	Pb	<i>Medicago truncatula</i> L. (Barrelclover)	10 $\mu$ M	Flavonoids	↑	(Zhang <i>et al.</i> , 2020)
7.	As (25 $\mu$ M)	<i>Camellia sinensis</i> L. (Tea)	100 $\mu$ M	Anthocyanins (CsCHS and CsANS)	↑	(Li <i>et al.</i> , 2021)
8.	Cd (50 $\mu$ M)	<i>Malva parviflora</i> (Mallow)	15, 50 $\mu$ M	PAL, total phenolics	↑	(Tousi <i>et al.</i> , 2020)

**Note:** PAL = Phenylalanine Ammonia Lyase; PPO = Polyphenol Oxidase; CHS = Chalcone Synthase; ANS = Anthocyanin Synthase

delay and decreased leaf area and biomass in *Arabidopsis*. Consequently, there is a characteristic reduction in expression levels of ent-kaurene synthase (a key enzyme in GA biosynthesis) (Lee *et al.*, 2019). Similarly, cytokinins and brassinosteroids (BRs) have been regulated by melatonin. Under drought stress in *Carya cathayensis*; zeatin (ZT), gibberellin A14 (GA14), 24-epibrassinolide (24-EBL), and JA are reported to be up-regulated by melatonin while ABA is down-regulated (Sharma *et al.*, 2020). In alleviation of Cd toxicity, the phytochelatin content, AsA-GSH cycle and, glyoxalase pathway have been improved by the combined effects of melatonin and SA in safflower seedlings (Amjadi *et al.*, 2021). The expressions of ABA biosynthetic genes (*CsNCED1* and *CsNCED2*) and its catabolic genes (*CsCYP707A1* and *CsCYP707A2*) are modulated by exogenous melatonin under chill stress in cucumber (Zhao *et al.*, 2017). In another study, it is found that the cold stress tolerance could be improved by melatonin influencing JA and H<sub>2</sub>O<sub>2</sub> accumulation (Li *et al.*, 2021). Likewise, the JA biosynthesis including lipoxygenase-related genes and metabolites such as linoleic acid and lecithin are induced by melatonin under copper stress (Hu *et al.*, 2020).

## 5.2. Melatonin influences secondary metabolites

An improved chromium stress tolerance has been shown in *P.ovata* seedlings by upregulating the gene expressions related to phenylpropanoid pathway, including *PAL* (phenylalanine ammonia-lyase) and *PPO* (polyphenol oxidase) (Kundu *et al.*, 2018). Melatonin also plays a key role in regulating the intermediate steps of phenylpropanoid pathway of polyphenol biosynthesis (Xu *et al.*, 2017; and Xu *et al.*, 2017) and flavonoid biosynthesis (Gao *et al.*, 2020). It also modulates few gene expressions that are responsible for secondary metabolite regulations in grape skin. These include *VvSTS1* (stilbene synthase), *VvF3H* (flavonoid 3'-hydroxylase), *VvDFR* (dihydroflavonol reductase), *VvMYB14*, and *VvACS1* (aminocyclopropane-1-carboxylate synthase1). Moreover, *VvACS1* has been activated by melatonin-induced *VvMYB14* resulting in the promotion of ethylene biosynthesis (Ma *et al.*, 2021). The transcription factors (*MYB*, *bHLH*, and *WD40*) for the anthocyanin biosynthesis have been over-expressed by melatonin (Zhang *et al.*, 2016). Independent of light conditions, the proanthocyanin (PA), anthocyanin, and flavonol contents are enhanced by melatonin in malus crabapple plants (Chen *et al.*, 2019). The expressions of genes related to melatonin (*TDC*, *SNAT*, and *ASMT*) and phenolic (*PAL*, *C4H*, *CHS*, *CHI*, and *STS*) biosynthesis are studied in presence of altered temperature and Cu stress. It is found that *ASMT* (which catalyzes the final step for melatonin biosynthesis) is mostly up-regulated than the rest of the genes of its pathway. Phenolic biosynthesis (including *C4H*, *CHI*, and *STS* genes) is over-expressed *PAL* expression is first increased and then decreased (Wang *et al.*, 2019). In Moldavian balm (*Dracocephalum moldavica*) plants, the flavonoids (anthocyanins), polyphenol compounds, and enzymes such as *PAL* and *PPO* are promoted by melatonin under average and critical drought stress (Naghizadeh *et al.*, 2019). The secondary metabolites (phenols, flavonoids, and anthocyanin) contents are increased by melatonin in addition to the gene expressions related to them (e.g., *PAL*, *CHS*). They are thus involved in limiting HO accumulation under Ni stress in tomato plants (Jahan *et al.*, 2020). It is reported that the iron stress is stabilized by melatonin through regulating the secondary metabolism and improving the phenols and flavonoids contents in cucumber. Also, different signaling molecules including silicon, salicylic acid, nitric oxide, polyamines, and abscisic acid have been enhanced (Ahmed *et al.*, 2020). In presence of Cu treatment, *TDC*, *SNAT*, and *ASMT* are significantly induced along with *C4H*, *CHI*, and *STS* genes (Wang *et al.*, 2019). Alkaline stress is alleviated by the interaction of melatonin with polyamine biosynthesis (Gong *et al.*, 2017). The increased levels of secondary metabolites (phenols, flavonoids, and anthocyanin) have been shown in melatonin-treated tomato seedlings to alleviate Ni toxicity (Jahan *et al.*, 2020). These reports help us to understand the various genes and pathways involved in the production of secondary metabolites in presence of melatonin to regulate the stress responses (Table 3).

## 6. Conclusion and future perspectives

A lot of evidence shows that phytomelatonin acts as a ubiquitous defensive molecule to alleviate stressful conditions. In this review, we summarized the impacts of melatonin on HM-induced responses in plants including regulation of melatonin biosynthesis, HM accumulation and translocation, ROS/RNS oxidative damages, and phytohormones and secondary metabolites mediated metal tolerance (Figure 2). Although several advances have been made to understand the key roles of melatonin against HM toxicity and other abiotic stresses, many questions still need attention. The metal transporters and chelators which detoxify the HMs are modulated by melatonin but have no clear information regarding their signaling mechanism. More studies are required to understand the role of melatonin in coping with the HM-induced excess ROS production

at chloroplast compartments and further protecting the photosystem reaction centers against free radicals. The molecular signaling between melatonin and other phytohormones such as IAA, ABA, ET, GA, SA, brassinosteroids, and JA is not clear. Likewise, the interaction between melatonin and secondary metabolites also needs critical observation as they might play a pivotal role in defense processes. Finally, further investigation should be done to know the effect of exogenous melatonin in the melatonin biosynthesis in plants under HM toxicity. Besides, more experiments are required to identify the phytomelatonin receptors and their downstream stress-responsive pathways to understand their proper functions. Melatonin-induced transcriptomic changes have been studied in bermudagrass, rice, *Arabidopsis*, soybean, and cucumber (Byeon *et al.*, 2014; Weeda *et al.*, 2014; Shi *et al.*, 2015; and Wei *et al.*, 2015). Structural Biology and Bioinformatics approaches would be helpful to understand more about the target genes and regulatory system. Many homologous genes that are responsible for melatonin biosynthesis have been cloned in different crop plants (Wang *et al.*, 2014; and Byeon *et al.*, 2016). Melatonin is an indole derivative and thus indole might be a target for future transgenic plants with increased melatonin concentration and improved abiotic stress tolerance. Such breakthroughs in crop production will improve agricultural production and general human health (Tan *et al.*, 2012).

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### Conflict of Interests

All authors declare no conflict of interests

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