

EXPLORING THE ROLE OF ABIOTIC ELICITORS IN THE BIOSYNTHESIS OF SECONDARY METABOLITES IN PEPPERMINT (*Mentha piperita* L.)

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ABSTRACT

Secondary metabolites are a large group of organic compounds generally biosynthesized by medicinal and aromatic plants, which have a wide range of uses in human life today. Peppermint (*Mentha piperita* L.) is widely used as a traditional medicinal plant worldwide due to its high content of secondary metabolites, including menthol, limonene, pulegone, rosmarinic acid, cinnamic acid, eriocitrin, narirutin and hesperidin. It has different medicinal and culinary uses, such as food flavoring and treating rheumatoid arthritis pain, sinusitis headache and breathing problems. Given the chemical synthesis of plant secondary metabolites under laboratory conditions is considerably expensive and complicated, some alternative methods have been developed. Applying abiotic elicitors such as UV-radiation, abiotic stresses, and phytohormones during peppermint cultivation is an effective strategy to modify secondary metabolite content and components. Therefore, in this review, the most important secondary metabolites of peppermint and their uses are first described, and the abiotic elicitors used to influence the secondary metabolites profile of peppermint and their reaction mechanisms are then explained.

Keywords: elicitation, essential oil, chemical elicitors, menthol, mint

INTRODUCTION

Plants produce a large group of organic compounds, called secondary metabolites, with generally low molecular weight (lower than 150 kDa), which, according to reports, have considerably low concentrations in plant tissues (lower than 1% of plant dry weight) [Abdi and Karami 2020]. To date, more than 100,000 secondary metabolites have been identified,

and more are still being identified [Haydari et al. 2019]. Secondary metabolites vary depending on the species and growth stage and play important ecological roles in plants. For instance, protection against herbivores and microbial agents, attraction of pollinators and improvement of plant-microbe symbiosis are some of the vital ecological roles of secondary metabolites [Abdi

and Karami 2020, Ahmad et al. 2022]. Currently, secondary metabolites are widely used in human life as medicine, biotic herbicides, flavoring agents, natural colors, pesticides, hallucinogenic substances (such as cocaine, heroin and morphine) and perfumes [Nazerieh et al. 2018, Abdi and Karami 2020]. There are three main pathways for the biosynthesis of secondary metabolites in plant tissues including: the mevalonic acid pathway, the malonic acid pathway and the shikimic acid pathway [Nazerieh et al. 2018, Haydari et al. 2019, Tabbert et al. 2022]. Secondary metabolites are generally grouped based on their biosynthesis pathway: nitrogen-containing secondary products (such as nicotine and polyamines), phenolic compounds (such as flavonoids), oxylipins (such as jasmonic acid) and terpenes (such as steroid alkaloids) are four main groups of plant secondary metabolites [Abdi and Karami 2020].

Peppermint (*Mentha piperita* L.) is a perennial herb belonging to the Lamiaceae family [Afkar et al. 2013], a natural hybrid from watermint (*Mentha aquatica* L.) and spearmint (*Mentha spicata* L.) [Askary et al. 2016]. Peppermint is widely used as a traditional medicinal plant worldwide due to its high content of secondary metabolites. Ancient Egyptian people cultivated this plant for different medicinal purposes, such as better food digestion and treatment of stomach ailments [Askary et al. 2016, Abdi and Karimi 2020]. Currently, several products with a wide range of medicinal and culinary uses, such as food flavoring and treating rheumatoid arthritis pain, sinusitis headache and breathing problems, are produced from this plant [Cappellari et al. 2020]. The aerial parts of peppermint are enriched sources of phenolic compounds, flavonoids, fatty acids, vitamins (A, C and B₆), nutrients (K, Ca, Mg, Fe, Mn, Zn and Cu) and salicylic acid. Essential oil is the most important phytochemical compound of this plant, mainly extracted from leaves through the steam distillation method. Monoterpenes such as menthol, menthyl acetate and menthone are considered the main components of peppermint essential oil [Nazerieh et al. 2018, Haydari et al. 2019, Cappellari et al. 2020, Tabbert et al. 2022]. Studies have shown that the ethanol extract of peppermint contains tannins and flavonoids, while glycosides, saponins and alkaloids are not extracted by methanolic extract [Nemati Lafmejani et al. 2018]. More than 40 phenolic compounds

such as rosmarinic acid, cinnamic acid, caffeic acid and salvianolic acid and also some flavonoid glycosides such as eriocitrin, narirutin, hesperidin, isorhoifolin and diosmin were extracted from the aerial parts of peppermint. The amount of phenolic and flavonoid compounds in aerial parts of peppermint has been reported to range from 2.8 to 17.8% and 0.71 to 3.86%, respectively [Nazerieh et al. 2018].

Given the chemical synthesis of plant secondary metabolites under laboratory conditions is considerably expensive and complicated, some alternative methods have been developed. Applying abiotic elicitors such as UV-radiation, abiotic stresses and phytohormones during medicinal plant cultivation is an effective strategy to change their secondary metabolite content and components. The word elicitor comes from elicit, which means extraction [Cappellari et al. 2020, Abdi and Karami 2020]. Elicitors are factors that directly or indirectly induce defensive changes in plants, leading to the activation of protective mechanisms and the biosynthesis of useful chemical compounds involved in plant adaptation to stress conditions. These factors are considered physical stimuli or chemical compounds with biotic or abiotic origins, which can induce different responses in plants, resulting in biosynthesis and accumulation of secondary metabolites in cells. Elicitors send some chemical messages to plant cells, which lead to physiological and morphological responses and the accumulation of phytoalexins. The antioxidant and defensive systems of plants are activated during the cell response to the elicitor's signals, leading to the expression of genes involved in secondary metabolite biosynthesis and accumulation [Merely et al. 2014, Abdi et al. 2018, Tabbert et al. 2022]. Using elicitors in limited amounts and low concentrations improves the biosynthesis of some compounds and generally reduces the time to achieve high amounts of metabolites. Elicitation is among the most effective practical methods to increase the biosynthesis of secondary metabolites in plant cells and tissues [Ahmad et al. 2022]. Elicitors may activate some new genes, leading to the production of new enzymes and activating new pathways, resulting in the accumulation of secondary metabolites. One of the most common groupings of elicitors is based on their nature, and according to that, the elicitors are divided into two different groups, including biotic and abiotic elicitors

(Fig. 1). Biotic elicitors are certain molecules from pathogens or host plants that can induce defensive responses. They are produced by plant enzymes on the cell membrane of microorganisms [Malik et al. 2020]. Moreover, biotic elicitors include some organic compounds produced by plant cells in response to various stimuli. Yeast extract, cell wall polysaccharides, oligosaccharides, proteins, glycoproteins and fatty acids are considered some of the most important biotic elicitors. Abiotic elicitors generally induce the production of phytoalexin in plant cells, and there are many reports on applying these factors to increase the secondary metabolite content of different plants [Nazerieh et al. 2018, Abdi et al. 2018, Cappellari et al. 2020, Tabbert et al. 2022].

The purpose of the current study was to investigate the roles of different abiotic elicitors in the biosynthesis and accumulation of different secondary metabolites in peppermint with the aim of better understanding the induction of biosynthesis pathways and the response mechanisms of plant cells to different abiotic stimuli.

MATERIALS AND METHOD

In this study, articles published, from 1999 to 2023, on the influence of abiotic elicitors on secondary metabolites of peppermint were also analyzed. Keywords, including “UV-radiation”, “heavy metals”, “chemical elicitors”, “Peppermint”, “*Mentha piperita*” and “secondary metabolites”, were searched for in the Science Direct, Scopus and Google search engines. Sixty-one articles were collected, and their contents were carefully analyzed for the purposes of this study.

RESULT

Most important secondary metabolites of peppermint

The amounts of monoterpenes, phenolic compounds and flavonoids are higher than the other secondary metabolites in peppermint [Atanassova et al. 2011, Bodalska et al. 2019, Mahendran and Rahman 2020] (Table 1). The most important monoterpenes extracted from peppermint leaves are menthol

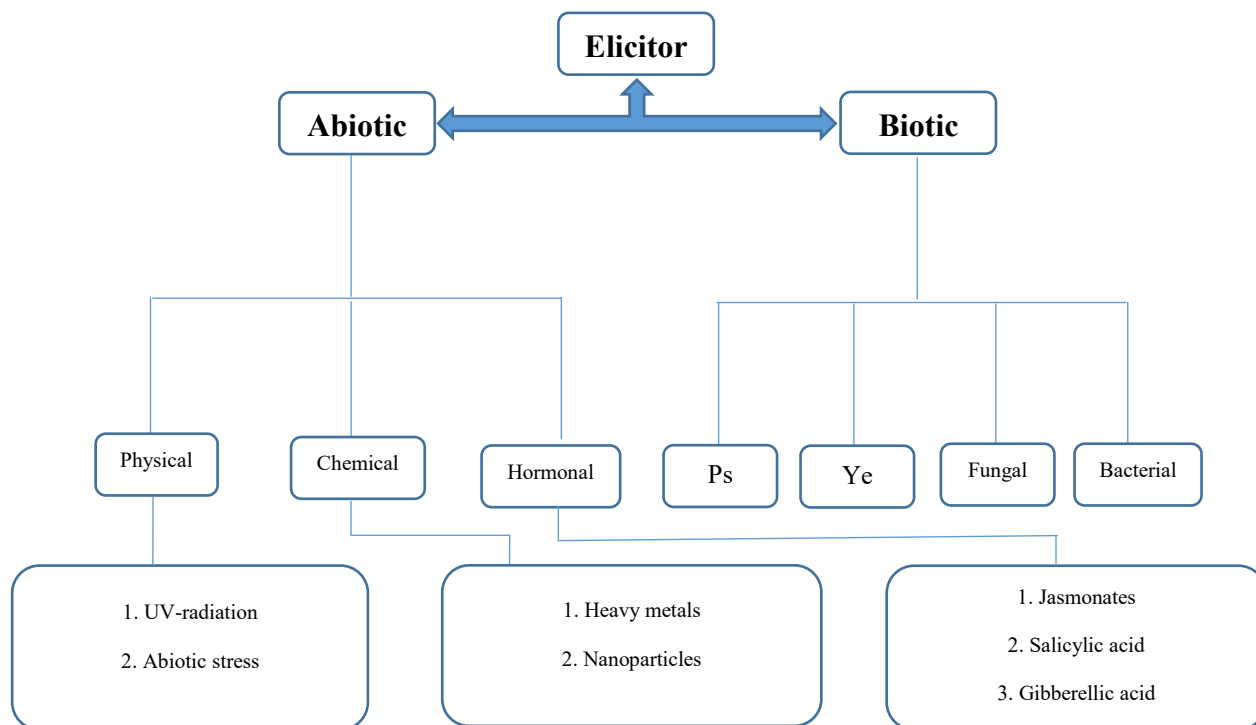


Fig. 1. Elicitors grouped based on their nature. Ps: polysaccharide, Ye: yeast extract

(30–55%), menthyl acetate (2.8–10%) and menthone (14–32%) [Croteau et al. 2005, Davis et al. 2005, Zhao et al. 2022]. Limonene (cyclic monoterpene), pulegone (monoterpene ketone), menthofuran, isomenthone (menthane monoterpene) and caron are the other monoterpenes found in peppermint leaves [Davis et al. 2005, Atanassova et al. 2011]. About 40 phenolic compounds, such as rosmarinic acid, cinnamic acid, caffeic acid and salvianolic acid, were extracted from the different aerial parts of peppermint [Sroka et al. 2005, Park et al. 2019, Naksawat et al. 2023]. Eriocitrin, narirutin, hesperidin, isorhoifolin, diosmin

Table 1. The most important secondary metabolites of peppermint

Secondary metabolite	Major compounds	Uses	Reference
Monoterpene	menthol	cosmetics, pain relief, improve the respiratory system	Mimica-Dukić et al. 2003, Croteau et al. 2005
	menthyl acetate	solvent for varnishes, lacquers, dry cleaning, stains, fats and nitrocellulose	Davis et al. 2005, Croteau et al. 2005
	menthone	treatment of stone formation in the gallbladder and liver	Davis et al. 2005, Croteau et al. 2005
	limonene	in medicinal ointments and creams penetrating the skin, fragrance, cleaner (solvent), and as an ingredient in household cleaning products, cosmetics, and personal hygiene products	Croteau et al. 2005, Bupesh et al. 2007, Zhao et al. 2022
	pulegone	flavoring agents, in perfumery, and in aromatherapy	Mahendran and Rahman 2020
	menthofuran	carminative and antispasmodic for esophageal spasm and irritable bowel syndrome	Davis et al. 2005, Croteau et al. 2005, Singh et al. 2015
	isomenthone	artificial flavorings in food, cosmetics, and pharmaceuticals, and even as pharmaceuticals themselves, e.g. camphor and eucalyptol	Davis et al. 2005, Croteau et al. 2005
Phenolic compound	caron	treatment and prevention of iron deficiency anemia; for preventing iron, folic acid and zinc deficiencies during pregnancy, breastfeeding, after surgery or in conditions of nutritional malabsorption	Davis et al. 2005, Croteau et al. 2005, Mahendran and Rahman 2020
	rosmarinic acid	treatment of inflammatory conditions such as arthritis, asthma, and atopic dermatitis	Aldoghachi et al. 2021
	cinnamic acid	flavorings, synthetic indigo, and certain pharmaceuticals	Sroka et al. 2005, Park et al. 2019
	caffeic acid	decreasing inflammation, preventing cancer, preventing toxicity associated with chemotherapy and radiation, preventing diabetes and premature aging, decreasing exercise-related fatigue	Lv et al. 2012, Park et al. 2019
Flavonoid	salvianolic acid	anti-oxidative activity	Lv et al. 2012
	eriocitrin	anti-atherosclerotic activity	Bodalska et al. 2019
	narirutin	anti-inflammatory and anti-oxidant	Bodalska et al. 2019
	hesperidin	treatment of blood vessel conditions such as hemorrhoids, varicose veins, and poor circulation (venous stasis)	McKay and Blumberg 2006, Bodalska et al. 2019
	isorhoifolin	antimutagenic	Bodalska et al. 2019
	diosmin	treatment of blood vessel disorders, such as hemorrhoids and chronic venous insufficiency	Bodalska et al. 2019, Soheilikhah et al. 2021
	Luteolin-glucuronide	depression-like and stress coping behaviors in sleep deprivation	Bodalska et al. 2019

and luteolin-glucuronide are the most important flavonoids extracted from peppermint leaves [Atanassova et al., 2011; Bodalska et al., 2019; Naksawat et al., 2023]. The amounts of phenolic compounds and flavonoids in peppermint aerial parts were reportedly about 2.8–17.8% and 0.71–3.86%, respectively [Ćavar Zeljković et al. 2021]. Atanassova et al. [2011] noted that the amount of phenolic compounds and flavonoids in aerial parts of peppermint is about 45.3 and 25.2 mg 100 g DW, respectively.

The reaction mechanisms of abiotic elicitors in peppermint

Abiotic elicitors act as stimulants and lead to morphological and physiological responses and the accumulation of phyto-alexins in different tissues of peppermint [Halder et al. 2019]. Research has shown that the treatment of peppermint with abiotic elicitors simulates a pathogen’s attack, leading to a set of defensive reactions and accumulation of secondary metabolites [Baenas et al. 2014]. Although the reaction

mechanisms of abiotic elicitors in peppermint are not yet clear, different mechanisms have been suggested, such as Ca²⁺ second messenger, factors involved in membrane integrity, inhibition or stimulation of intra- and inter-cellular pathways and changes in osmotic factor amounts and activity [Afkar et al. 2013, Askary et al. 2016, Nazerieh et al. 2018, Haydari et al. 2019, Abdi and Karami 2020, Ahmad et al. 2022]. Based on reports, the elicitors bind to membrane receptors, resulting in a change in cytosol pH and biosynthesis of reactive oxygen species (ROS) and increasing their activity rate, leading to up-regulating of the expression of genes involved in defensive responses [Afkar et al. 2013, Haydari et al. 2019]. Biological signals produced by abiotic elicitors induce the synthesis of the second messengers in plant cells, leading to kinase protein biosynthesis and subsequently enhancing secondary metabolite accumulation [Haydari et al. 2019]. Based on the studies conducted, the reaction mechanism of abiotic elicitors in peppermint can be explained based on Figure 2 [Afkar et al. 2013, Abdi

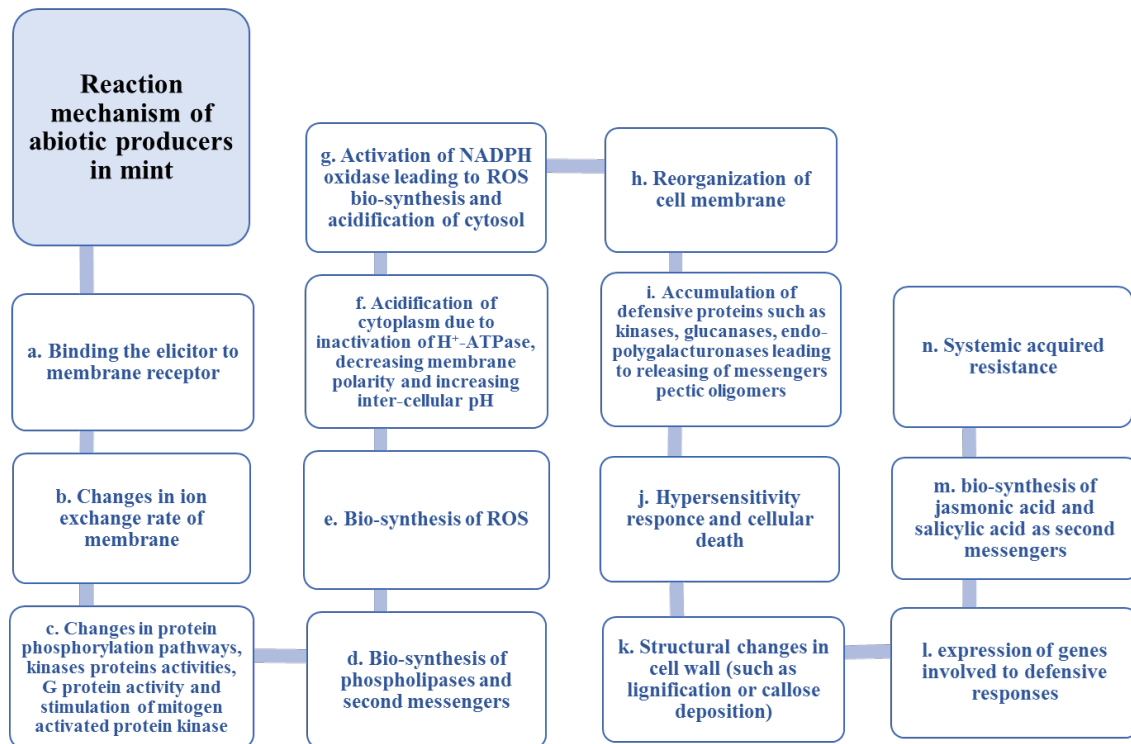


Fig. 2. The reaction mechanism of abiotic elicitors in peppermint; NADPH: nicotinamide adenine dinucleotide phosphate, ROS: reactive oxygen species

et al. 2018, Cappellari et al. 2020, Abdi and Karami 2020, Tabbert et al. 2022, Ahmad et al. 2022].

Effect of abiotic elicitors on secondary metabolites of peppermint

Physical elicitors. *UV-radiation.* Industrial crops such as peppermint show considerable metabolic changes in response to changes in environmental factors such as light quality and quantity [Farooqi et al. 1999, Croteau et al. 2005]. According to conducted studies, UV-B (280–315 nm), UV-A (315–400 nm) and PAR, meaning Photosynthetic Active Radiation (400–700 nm), as well as their respective ratios and interactions, are the most important radiation factors that can significantly influence secondary metabolite biosynthesis in peppermint [Maffei et al. 1999, Croteau et al. 2005, Behn et al. 2010, Dolzhenko et al. 2010, Mahendran and Rahman 2020, Tabbert et al. 2022].

UV-B radiation can affect the essential oil content of peppermint, and according to Behn et al. [2010], applying UV-B at 290 nm leads to a remarkable increase in peppermint essential oil content. Based on reports, UV-B radiation can remarkably affect the monoterpene content of peppermint leaves. The absence of UV-B radiation during the growth and development of the peppermint plant leads to lower menthol and higher menthone content, resulting in a significant reduction in oil quality (menthol is one of the main final products of a menthone reduction reaction catalyzed by menthone reductase enzyme) [Behn et al. 2010, Dolzhenko et al. 2010]. The influence of UV-B radiation on essential oil quality and content is correlated with the growth stage of the pepper plant. Measurements clearly demonstrate that the effect of UV-B radiation on essential oil components of peppermint is more pronounced in the flowering stage, while this effect is lower in the vegetative stage and is not significant during bud formation [Behn et al. 2010]. The results of some studies have shown that UV-B radiation can affect the monoterpene and oil quality of peppermint in interactions with PAR radiation. In low PAR conditions (regions with mostly cloudy weather), using supplemental UV-B radiation can increase secondary metabolites, monoterpene content and oil quality of peppermint leaves, while when the PAR radiation is sufficient, changes in UV-B radiation have no significant effect on oil quality or secondary

metabolite contents [Croteau et al. 2005, Mahendran and Rahman 2020]. The effect of UV-B radiation on the secondary metabolites of peppermint could be induced by changes in gene expression, enzymatic activities and defense responses [Saharkhiz and Goudarzi 2014]. Supplemental UV-B radiation in peppermint cultivation under greenhouse and open field conditions up-regulated the expression of five genes, including *dxs* (involved in early steps of terpenoid biosynthesis), *gpps* (involved in monoterpenes biosynthesis), *mr* (the most important gene involved in menthol biosynthesis) and *fpss* (involved in the biosynthesis of essential isoprenoids such as sterols and brassinosteroids, cytokinins, ubiquinone, dolichols, and prenylated proteins and some sesquiterpenes such as E- β -caryophyllene and germacrene-D); see Figure 3. Generally, this supplemental light treatment led to an increase in limonene, 1,8-cineol, E-(β)-ocimene, sabinene hydrate, linalool, menthone, menthofuran, pulegone, piperitone, piperitenone, (E)- β -caryophyllene and germacrene D. In addition, the content of eriocitrin, kaempferol 7-O-rutinoside and hesperidin is considerably increased in peppermint by induction of UV-B treatment [Dolzhenko et al. 2010].

As mentioned, UV-A (360 nm) radiation could be effective on peppermint metabolites. The results of a study regarding the influence of UV-A radiation on peppermint secondary metabolites demonstrated that the time of light treatment induction is effective in determining metabolite content and composition. Using supplemental UV-A radiation during the days led to a remarkable increase in phenolic compounds, essential oil content and menthol and menthofuran content, although light treatment during the nights led to the appearance of shade-avoidance syndrome, resulting in lower phenol, essential oil and menthol content. UV-A modulates the essential oil content of peppermint by regulation of enzymatic activity (menthone reductase enzyme) and gene expression (mainly the *mr* gene) [Maffei et al. 1999].

Abiotic stress. Research demonstrated that abiotic stresses such as drought stress, salt stress and heat stress change the content and ratio of the secondary metabolite components in medicinal plants [Charles et al. 1990, Khorasaninejad et al. 2011, Heydari et al. 2018, Alhaithloul et al. 2019, Hosseini et al. 2021]. Therefore, induction of abiotic stresses at low levels

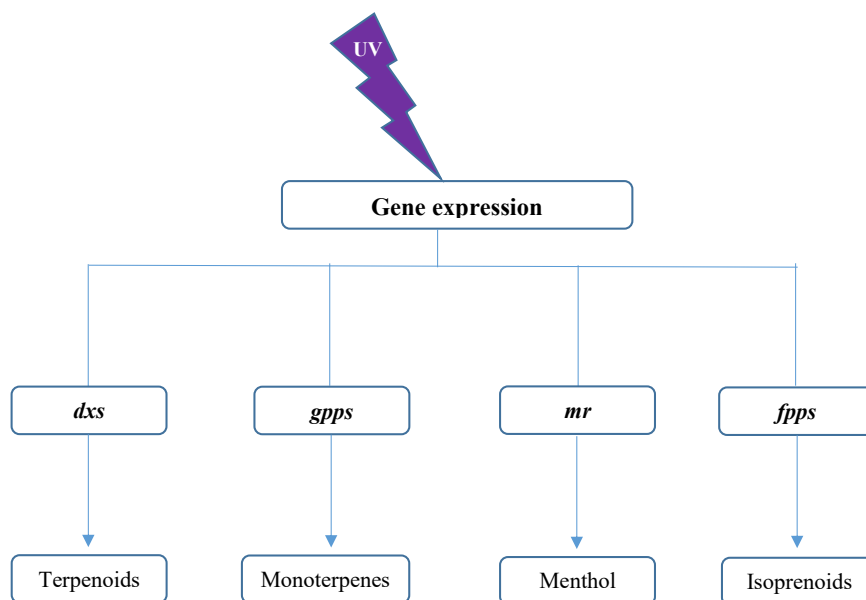


Fig. 3. The UV radiation effect on gene expression and metabolite biosynthesis in peppermint

during different stages of preharvest could be considered a widely used strategy to change the amounts of desired compounds in medicinal plants. The positive effects of abiotic stresses on increasing the amounts of phytochemical compounds such as phenolic acids, tannins and flavonoids in different tissues of horticultural plants have been reported in several studies [Aziz et al. 2008, Khorasaninejad et al. 2011, Roodbari et al. 2013, Li et al. 2014, Fathi et al. 2020]. Increasing secondary metabolites in response to environmental stresses is one of the plant's defensive mechanisms to alleviate the adverse effects of stresses [Hosseini et al. 2021]. Several studies have shown that the influence of stresses on the metabolites of medicinal plants is strongly correlated with stress severity. High levels of abiotic stresses increase secondary metabolite contents and can disrupt the pathways of metabolite biosynthesis by inducing negative effects on the plants' enzymatic and antioxidant activities and altering gene expression [Khorasaninejad et al. 2011, Li et al. 2014, Fathi et al. 2020].

Cultivation of peppermint under a deficit irrigation strategy (50% of field capacity) will cause a significant decrease in some important morphological characteristics such as plant height, leaf number and leaf

area index, leading to lower photosynthetic rate, dry matter and accumulation rate of secondary metabolites in plant tissues. Total phenolic compounds and flavonoid contents of peppermint leaves increased by 34.8% and 43.6%, respectively, when irrigation was carried out with 70% of field capacity [Khorasaninejad et al. 2011]. Increasing the ROS accumulation rate in plant cells induced by drought stress leads to increasing phenolic compounds and flavonoids as antioxidant agents [Aziz et al. 2008, Khorasaninejad et al. 2011, Roodbari et al. 2013]. Phenolic compounds act as antioxidants by donating electrons to peroxidase enzymes and detoxifying hydrogen peroxide [Li et al. 2014]. Under moderate levels of drought stress, the highest increase rate in phenolic compounds and flavonoids was observed in rosmarinic acid and hesperidin. Drought stress also caused the production of phenolic and flavonoid compounds such as coumaric acid, luteolin, quercetin, naringenin, and vanillin in peppermint, which was not observed in plants grown under normal conditions [Charles et al. 1990, Khorasaninejad et al. 2011]. It seems that the induction of drought stress leads to the expression of some new genes that lead to the biosynthesis of new phenolic compounds and flavonoids in peppermint. Studies

have shown that drought stress is generally a negative factor for peppermint menthol content [Khorasaninejad et al. 2011, Abdi et al. 2018]. Based on reports, drought stress, even in low severities, reduces menthol content in different tissues of peppermint [Abdi et al. 2020]. Measurements have shown that this negative effect comes from the influence of drought stress on *mr* gene expression. Down-regulation of *mr* gene expression will occur in water deficit conditions, and there is a strong negative correlation between drought stress severity and expression level of *mr* gene [Croteau et al. 2005, Davis et al. 2005]. However, the results of some studies have shown that when drought stress, along with the other elicitors such as salt stress, nanoparticles and hormonal agents, is induced on peppermint, the influence of drought stress on essential oil content and components is generally determined through the interaction between the factors. Mostly low severities of drought stress in interaction with salt stress, nanoparticles, methyl jasmonate and salicylic acid can enhance peppermint essential oil content and quality [Alhaithloul et al. 2019, Abdi et al. 2018, Abdi et al. 2020].

Salt stress is one of the abiotic stresses which can affect secondary metabolite biosynthesis in peppermint [Aziz et al. 2008]. Different reports have been published about the effect of salinity on the secondary metabolites of peppermint [Roodbari et al. 2013, Li et al. 2014, Fathi et al. 2020, Hosseini et al. 2021]. Some studies have shown that inducing salinity stress during peppermint cultivation can increase the content of secondary metabolites in the aerial parts of the plant. However, other research demonstrated that salinity negatively impacts the biosynthesis of essential oil and other secondary metabolites in peppermint. According to Roodbari et al. [2013], growing peppermint under salinity conditions leads to more energy consumption by plant cells leading to lower carbon content that is available for the biosynthesis of secondary metabolites. Moreover, changing the hormonal ratios (abscisic acid to cytokinin) and lowering the amounts of cytokinin in plant aerial parts under salinity conditions are considered other important reasons for the lower biosynthesis rate of secondary metabolites. Studies on the influence of salinity on essential oil content and constituents demonstrated that doubling the salinity level led to a 48.28% decrease in essential

oil content. A 55.18% decrease was also observed in essential oil yield when the salinity level increased by three times. Lower levels of salinity increased menthol content, but increased salinity levels to concentrations higher than 3 g L^{-1} NaCl drastically decreased menthol content in peppermint essential oil [Fathi et al. 2020]. Assessment of *mr* enzyme activity showed that its activity rate is strongly negatively correlated with the severity of salt stress. Pulegone, isomenthone, linalool and myrcene were the other essential oil components whose concentrations exhibited a negative correlation with salinity level [Hosseini et al. 2021]. Peppermint essential oil is mostly produced by epidermal oil glands, which are carbon heterotrophic. Therefore, a lower photosynthetic rate induced by higher levels of salinity leading to lower available carbon could be considered the main reason for lower essential oil content under salinity conditions [Roodbari et al. 2013, Li et al. 2014]. Similar to the other abiotic stresses, salinity generates high levels of excited energy and ROS, leading to damage to the cell membrane and a higher electrolyte leakage rate. Peppermint cells were found to produce increased levels of phenolic compounds and flavonoids in response to salinity, which acts as an abiotic stimulus. This response serves as a defensive mechanism to combat aggressive agents [Aziz et al. 2008]. Increased biosynthesis of polyphenols, especially in photosynthetic structures, and a rise in the amount and activity of glutathione s-transferase enzyme (this enzyme is involved in the transfer of flavonoids to vacuole) were recorded in peppermint grown under saline conditions [Li et al. 2014]. Moreover, studies have shown that induction of salinity stress at low levels increases the PAL enzyme activity in peppermint cells. However, increasing stress severity not only reduces PAL enzyme activity but also leads to a higher polymerization rate of soluble phenolic compounds, leading to a reduction in total phenol content [Roodbari et al. 2013, Li et al. 2014, Fathi et al. 2020]. Finally, the current study showed that the effect of salinity and drought stress on the biosynthesis of secondary metabolites in peppermint tissues drastically depends on the stress severity rate (Fig. 4).

Alhaithloul et al. [2019] demonstrated that cultivation of peppermint under heat stress conditions causes the accumulation of glycine betaine, inositol and mannitol in plant cells. The maximum accumulation

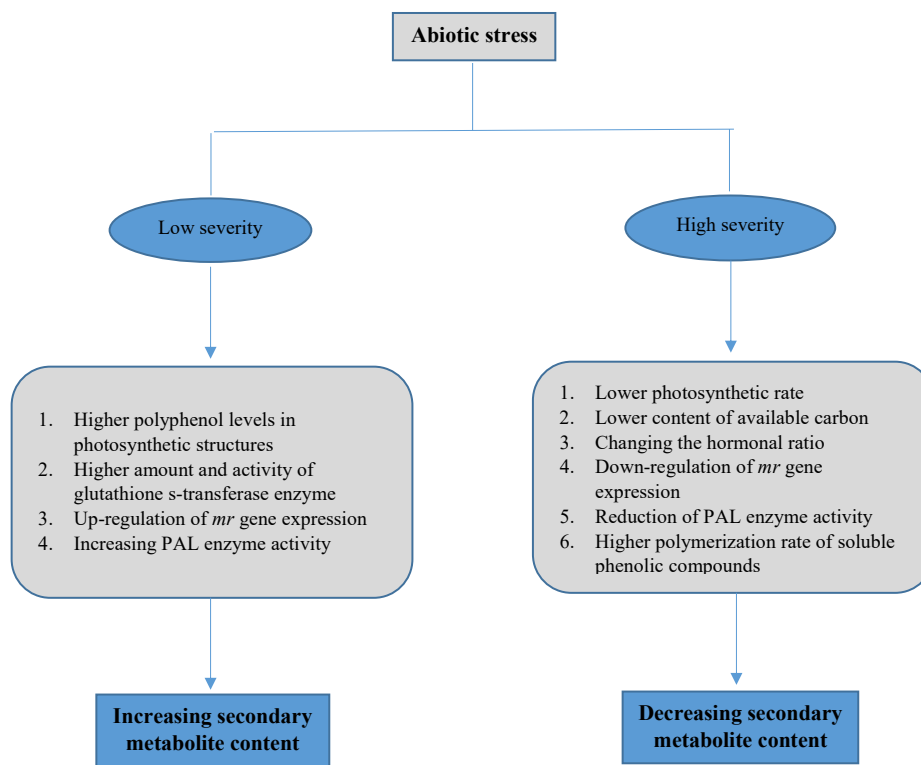


Fig. 4. The influence of abiotic stresses on the biosynthesis of secondary metabolites in peppermint is strongly correlated with the severity of stress

rates of these compounds were observed when the heat stress was induced with low levels of drought stress. A molecular assessment also showed that the expression rate of genes involved in the shikimic acid pathway and biosynthesis of some alkaloids, such as anthocyanins and lignin, up-regulated by induction of heat stress during peppermint cultivation. The effect of heat stress on the *mr* gene is similar to that of drought stress, which indicates a negative correlation between heat stress severity and the *mr* gene expression rate. Studies have shown that cultivation of peppermint under temperatures of 23–25 °C leads to the highest *mr* enzyme activity rate and accumulation of menthol in peppermint aerial parts [Heydari et al. 2018, Alhaithloul et al. 2019].

Chemical elicitors. Heavy metals. Accumulation of heavy metals in agricultural soils through irrigation with contaminated wastewater not only causes soil pollution but also significantly reduces food

quality and safety [Valko et al. 2005, Nazerieh et al. 2018, Nemati Lafmejani et al. 2018, Azimychetabi et al. 2021, Ahmad et al. 2022]. Among the heavy metals, Cd and Pb have been studied and investigated more than the others due to their high stability in the environment [Valko et al. 2005, Azimychetabi et al. 2021]. According to reports, plants need these elements in low concentrations, and accumulation of high amounts of heavy metals in soil can negatively affect seed germination, seedling growth and both the qualitative and quantitative characteristics of mature plants [Nemati Lafmejani et al. 2018, Azimychetabi et al. 2021, Ahmad et al. 2022]. According to Zheljzkov et al. [2006], the total yield of peppermint was not significantly changed when cultivated in soil enriched with heavy metals (Cd, Pb and Cu), although the menthol content was considerably decreased. In addition, the essential oil of peppermint plants did not contain any heavy metals. Menthol, as the most important sec-

ondary metabolite of peppermint, is bio-synthesized in eight steps, including the production of geranyl diphosphate from isopentenyl pyrophosphate, which is catalyzed by geranylgeranyl diphosphate synthase enzyme, the biosynthesis of limonene by the activity of limonene synthase enzyme, the biosynthesis of menthone from limonene through several oxidation, reduction, and polymerization reactions, and finally, the biosynthesis of menthol from the menthone by the activity of the menthone reductase enzyme [Croteau et al. 2005]. Thus, menthol is produced through a multi-enzyme pathway, and changes in the activity rate of each enzyme can affect the menthol biosynthesis rate in peppermint. Studies have shown that low concentrations (lower than 50 ppm) of some heavy metals such as Mn, Cd and Pb can up-regulate the expression of the limonene synthase gene, leading to more amounts of precursor for menthone and, subsequently, menthol biosynthesis. However, high concentrations (more than 100 ppm) of these heavy metals can reduce menthol biosynthesis rates by down-regulating the expression of the menthone reductase enzyme [Valko et al. 2005]. Nazerieh et al. [2018] reported that the influence of Se on secondary metabolites of peppermint is significantly related to the concentration of metals used in treatments. Based on their results, low concentrations of Se can increase total phenolic content and menthol in peppermint leaves by enhancing phenylalanine ammonia lyase (a key enzyme of phenylpropanoid metabolism) and menthone reductase enzyme activities. Cultivation of peppermint in soils with low concentrations of Cd and Pb did not affect the percentage of essential oil in the leaves. However, when the concentration of these heavy metals was increased to 100 ppm, both the quantity and quality of the leaf essential oil were reduced. This reduction may be linked to a decrease in the total dry matter of the leaves [Valko et al. 2005]. Since terpenoids produced by leaf epidermal cells are considered consumers of carbon produced through photosynthesis, the essential oil biosynthesis rate in peppermint leaves is remarkably correlated with the continuous production of photosynthetic carbon. High amounts of heavy metals can negatively affect essence production through photosynthesis rate reduction [Nazerieh et al. 2018, Lafmejani et al. 2018, Azimychetabi et al. 2021]. Studies on the influence of Cd on secondary metabolites

of peppermint have shown that cultivating this crop in soils with high concentrations of Cd leads to decreasing menthol content and increasing menthofuran and pulegone contents. Increasing Cd levels in a substrate are followed by down-regulation of menthone reductase and pulegone reductase enzyme expression and up-regulation of menthofuran synthase enzyme expression [Azimychetabi et al. 2021]. Ahmad et al. [2022] reported that the reduction of secondary metabolites content in aerial parts of peppermint induced by cultivation in soils enriched with heavy metals is correlated with changes in activities of two vital regulatory enzymes, phenylalanine ammonia-lyase and deoxy-D-xylulose-5-phosphate reductoisomerase. These two enzymes are key factors in the shikimic acid pathway (an important pathway for phenolic compound biosynthesis) and the methylerythritol 4-phosphate pathway (an important pathway for terpenes biosynthesis); see Figure 5.

Nanoparticles. Nanoparticles of different elements have been used as chemical elicitors in different crops and cultivation processes to enhance qualitative and quantitative characteristics [Abdelsalam et al. 2023]. Nanoparticles can easily go through the pores of the cell wall and enter the plasma membrane due to their tiny size. Thus, their biological efficiency could be higher than elements of larger sizes [Ahmad et al. 2018]. Using these factors at different physiological steps, from seed germination to flowering and fruiting, has been reported in different crops such as wheat, rice, apple, basil, *etc.* [Ahmad et al. 2010, Ali et al. 2016, Jankovskis et al. 2022]. Considerable changes in enzymatic activity and gene expression leading to better growth, development and defensive responses were observed after applying these elicitors during crop cultivation [Nemati Lafmejani et al. 2018]. To date, the influence of iron, copper, titanium dioxide and iron oxide nanoparticles on secondary metabolites of peppermint has been investigated.

Using titanium dioxide nanoparticles at a concentration of 150 mg L⁻¹ increased the essential oil content of peppermint by 39.4% compared to a control. This treatment also led to a higher menthol content (9.6% higher than the control) in treated plants. Treatment of peppermint with titanium dioxide nanoparticles leads to higher photosynthetic pigment content (chlorophyll a, chlorophyll b, total chlorophyll content, carotenoids

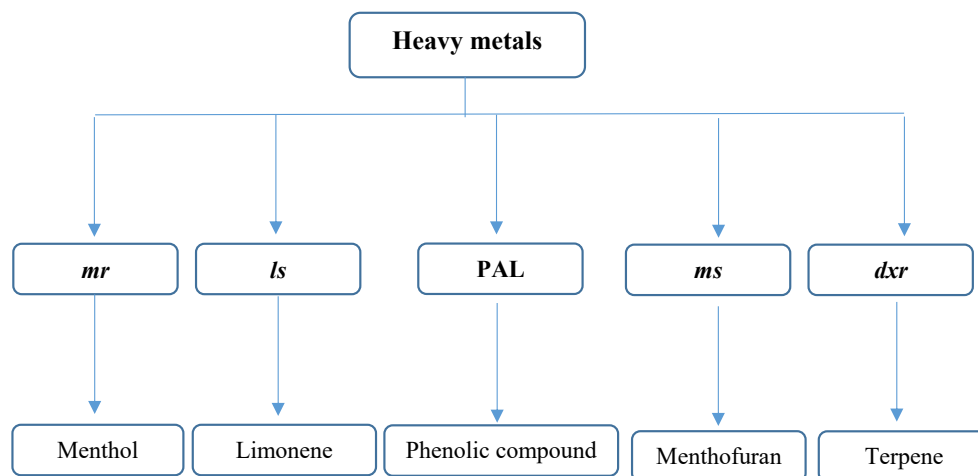


Fig. 5. The influence of heavy metals on the activity of enzymes involved in secondary metabolite biosynthesis in peppermint; *mr*: menthone reductase, *ls*: limonene synthase, PAL: phenylalanine ammonia-lyase, *ms*: menthofuran synthase and *dxr*: deoxy-D-xylulose-5-phosphate reductoisomerase

and anthocyanin) and subsequent photosynthetic rate. Both an increase in *mr* gene expression and a higher activity rate of menthone reductase enzyme were observed in treated plants with titanium dioxide nanoparticles, leading to higher menthol content compared to the control. Increased growth rate, photosynthesis rate, expression of terpene biosynthesis enzymes and density and diameter of oil glands were considered the most important reasons for the enhanced production of secondary metabolites in peppermint treated with titanium dioxide nanoparticles [Ahmad et al. 2018].

According to Nemat Lafmejani et al. [2018], there were no significant differences between the concentrations of 0.5, 1 and 1.5 g L⁻¹ of iron nanoparticles in terms of their influence on monoterpene content in peppermint. These treatments increased the essential oil content of peppermint by 50–60%. The concentrations of menthone and menthol in treated plants also increased by more than 65% and 34%, respectively, compared to control. Iron nanoparticle foliar application at the flowering stage increased the menthofuran content in peppermint leaves more than twice.

Using iron oxide nanoparticles at 30 µm concentration during peppermint vegetative growth and flowering stage led to a higher essential oil content of treated plants (3.56%) compared to the control (2.19%). Studies have shown that iron oxide nanoparticles can influ-

ence the types and proportions of major components of peppermint essential oil. When the peppermint plants were sprayed with iron oxide nanoparticles at a concentration of 30 µm, menthone (49.67%), menthol (22.19%), 1,8-cineol (7.90%), pulegone (2.86%), menthofuran (2.84%), cis-sabinene hydrate (2.52%) and germacrene D (1.69%) were the major components of peppermint essential oil. Furthermore, the proportion of menthone increased and that of pulegone doubled. Increasing Fe concentration from 10 to 30 µm resulted in a higher total essential oil content, however, its quality decreased due to a reduction in menthol content and an increase in menthofuran content [Askary et al. 2016].

Copper, as an essential micronutrient, plays vital roles in different physiological processes such as cell wall metabolism, biosynthesis of regulatory proteins, mitochondrial respiration and biosynthesis of phytohormones. Copper acts as a cofactor for the activity of several enzymes involved in some of the most important plant physiological processes [Lafmejani et al. 2018]. The effect of copper nanoparticles on secondary metabolites of peppermint is correlated with the nutrient concentration used in the treatment. Studies have shown that using high concentrations of copper reduces the total secondary metabolite content of some crops, such as beans, wheat, and peppermint, because

of its toxicity effects and growth inhibition. According to reports, foliar application of copper nanoparticles at the concentration of 1 g L⁻¹ during the flowering stage of peppermint could be a widely used technique to increase the essential oil content of peppermint. However, exceeding this concentration may reverse the increase in essential oil production [Nemati Lafmejani et al. 2018]. Nemati Lafmejani et al. [2018] reported that using copper nanoparticles during the reproductive growth of peppermint will increase the leaf menthol and phenolic compound contents by 15 and 18%, respectively.

Hormonal elicitors. Studies have shown that hormonal elicitors can affect gene expression, enzymatic pathways and biosynthesis of metabolites in plants. Hormonal elicitors are used during crop cultivation for different purposes, such as increasing productivity rate, enhancing tolerance rate against biotic and abiotic stresses or improving qualitative characteristics [Soleymani et al. 2015, Çoban et al. 2016, Soleymani et al. 2017, Abdi et al. 2018, Abu El-Leel et al. 2021]. Based on reports, methyl jasmonate, salicylic acid, gibberellic acid, and melatonin are some hormonal elicitors that can significantly affect the secondary metabolite content and essential oil quality of peppermint.

Methyl jasmonate. Foliar application of methyl jasmonate at 0.1% concentration at the vegetative stage can induce alteration of gene expression pattern in peppermint. Twenty-one out of 33 transcript-derived amplicons were the new transcripts. This change in gene expression pattern induced by methyl jasmonate application led to a significant increase in volatile oil percentage. The total concentration of monoterpene hydrocarbons increased by 6.85% in treated plants compared to control. The concentration of oxygenated monoterpenes also increased by 4.42% in treated plants compared to non-treated plants. Increasing menthol content and decreasing menthone content and menthofuran gene expression (which produces higher quality essential oil) were the other effects of methyl jasmonate application during the vegetative growth of peppermint [Abu El-Leel et al. 2021]. As mentioned, methyl jasmonate application can induce changes in gene expression patterns. Genes such as *pr*, *mfs* and *ls* were considered the most important genes involved in changes in the content of monoterpenes induced

by methyl jasmonate application. Generally, *pr*, *mfs* and *ls* are key genes in the monoterpene biosynthesis pathway in peppermint [Krzyzanowska et al. 2012]. Previous studies have shown that up-regulation of the expression of these genes in response to methyl jasmonate application will result in higher menthol content [Abu El-Leel et al. 2021]. The expression of these genes will lead to higher amounts and activities of some key enzymes involved in menthol biosynthesis in peppermint. Moreover, increasing the pulegone and menthofuran biosynthesis was observed after the up-regulation of the expression of *pr* and *mfs* genes induced by methyl jasmonate application [Afkar et al. 2013, Soleymani et al. 2017]. The results have demonstrated that the response of *pr* and *ls* genes to methyl jasmonate application is faster than that of *mfs* gene, which means that the expression level of *mfs* gene might be related to the expression of two other genes. This indicates that the effect of methyl jasmonate application on monoterpenes of peppermint is not only induced by changes in transcript levels but also by varying the rates of expression among different genes. This has a significant impact on changes in secondary metabolites [Afkar et al. 2013].

Salicylic acid. Salicylic acid is a widely used chemical elicitor to improve the metabolite profile, nutritional value and antioxidant characteristics of peppermint [Abdi et al. 2020]. For instance, Figueroa-Pérez et al. [2015] reported that the application of salicylic acid at 1 mM concentration increased the total phenolic content of treated plants, and some secondary metabolites such as sinapic acid, rutin and naringin were detected only in salicylic acid-treated plants. Saharkhiz and Goudarzi [2014] reported that using salicylic acid at a concentration of 150 mg L⁻¹ significantly increased the essential oil content compared to untreated plants. In particular, salicylic acid treatment mostly increased menthone (15.8–18.1%) and menthol (46.3–47.4%) content. The effect of salicylic acid on the essential oil of peppermint is correlated with the concentration of treatment. Previous studies have shown that using salicylic acid at a concentration of ≤2 mM led to increasing total essential oil content by 1–2%, but on the other hand, increasing the concentration of the treatment chemical up to 2 mM reduced the essential oil content and quality [Cappellari et al. 2019]. However, under water stress conditions, using salicylic acid

treatment at concentrations higher than 2 mM resulted in increased essential oil contents (more than 2-fold compared to control) [Abdi et al. 2020]. Increasing phenolic compounds, especially cinnamic acid and salvianolic acid, which occur through an increase in phenylalanine ammonia-lyase enzyme activity, as well as higher flavonoid levels, particularly eriocitrin and narirutin, induced by salicylic acid treatment, were also reported in various studies [Saharkhiz and Goudarzi 2014, Haydari et al. 2019, Abdi et al. 2020]. The influence of salicylic acid treatment on the expression of genes involved in monoterpenes biosynthesis in peppermint demonstrated that using treatments at a concentration below 1 mM had no significant effect on *ls* and *pr* gene expression, while increasing the treatment concentration led to up-regulating gene expressions and higher monoterpenes biosynthesis rate in peppermint [Cappellari et al. 2019]. Figueroa-Pérez et al. [2015] reported that treatment of peppermint plant with salicylic acid at a concentration of 0.5 mM can increase some triterpene and steroid saponins (such as phytolaccagenic acid, phytolaccagenic acid, hederagenin, serjanic acid, campesterol, stigmasteryl and sitosterol) and the concentration of some alkaloids (such as choline, trigonelline, nicotinic acid, sibiricine, vinblastine, vindoline, catharanthine and vinleurosine). Based on the results of these authors, changes in the saponin and alkaloid profiles induced by salicylic acid treatment led to improving anti-diabetic effects of peppermint. To develop systemic acquired resistance (SAR), a plant must generate a signal in the pathogen-inoculated tissue that travels (presumably through the vasculature) to the uninoculated portions of the plant, in which it signals defense responses. Radio-tracer studies in tobacco and cucumber initially indicated that some of the salicylic acid (SA) in systemic leaves was synthesized in the inoculated leaf, raising the possibility that SA was the mobile signal [Mölders et al. 1996, Shulaev et al. 1995]. Consistent with this possibility, pathogen-induced SA was shown to move through the apoplast before being loaded to phloem in *Arabidopsis* [Lim et al. 2016], and SA was detected in phloem sap in pathogen-infected plants [Lim et al. 2016, Métraux et al. 1990, Mölders et al. 1996, Yalpani et al. 1991]. However, analyses of chimeric tobacco generated by grafting combinations of wild type (wt) or SA-deficient rootstocks and scions (the

upper half of the plant) revealed that plants containing a wt scion developed SAR even if the rootstock was SA-deficient. By contrast, plants containing an SA-deficient scion failed to develop SAR, regardless of the rootstock [Pallas et al. 1996, Vernooij et al. 1994]. Overall, these studies suggest that SA accumulation is required in uninoculated tissues to signal SAR, but SA is not likely the critical mobile signal.

Over the years, efforts to identify the mobile SAR signal have identified several candidates. The first SAR signal to be identified was the SA derivative MeSA. This finding was rapidly followed by the discovery that other compounds, including a nine-carbon dicarboxylic acid azelaic acid (AZA), glycerol-3-phosphate (G3P) or a G3P-dependent factor, the abietane diterpenoid dehydroabietinal (DA), and the lysine (Lys) derivative pipecolic acid (Pip), also are mobile inducers of SAR. In addition, SAR signaling mediated by some of these small metabolites appears to depend on one or both the lipid transfer protein (LTP) defective in induced resistance 1 (DIR1) and the LTP-like protein azelaic acid-induced 1 (AZI1). Since these signals and the complex network through which they interact have been the subject of several reviews published to date [Dempsey and Klessig 2012, Shah and Zeier 2013, Shah et al. 2014, Singh et al. 2017], only some of the more recent findings will be summarized here.

Genetic, molecular, and biochemical analyses have led Pradeep Kachroo and colleagues to propose that SAR is activated by parallel pathways mediated by SA and AzA/G3P [Singh et al. 2017, Wendehenne et al. 2014]. In this model, pathogen infection leads to the accumulation of SA and nitric oxide (NO), which triggers the accumulation of ROS via an amplification loop (Fig. 3). ROS, in turn, generate AzA from precursor C18 unsaturated fatty acids (FAs). AzA then induces the synthesis and accumulation of G3P, which travels via the symplast to the phloem and subsequently induces SAR, in conjunction with SA, in the systemic tissue [Lim et al. 2016, Wang et al. 2014, Yu et al. 2013]. DIR1 and AZI1, which interact with each other as well as themselves, impact this pathway by forming a positive feedback loop with G3P [Yu et al. 2013]. Since pathogen-induced accumulation of AzA and G3P as well as SAR were compromised in *Arabidopsis* mutants that lack the ability to synthesize the galactolipids monogalactosyldiacylglycerol (MGDG)

or digalactosyldiacylglycerol (DGDG), it was further hypothesized that AzA is generated via oxidation of C18 unsaturated FAs on MGDG and DGDG lipids, rather than free FAs [Gao et al. 2014]. Interestingly, the DGDG-defective mutant *dgd1* but not the MGDG-defective mutant *mgd1* failed to accumulate NO or PR-1 transcripts after pathogen infection; *dgd1* plants also displayed reduced accumulation of free SA and SAG in pathogen-inoculated leaves and reduced free SA accumulation in the systemic leaves. Thus, DGDG and MGDG appear to have additional, distinct functions that impact different steps of the SAR signaling pathway [Klessig et al. 2018].

Gibberellic acid. Gibberellic acid, as a phytohormone and plant growth regulator, is recognized as an important effective factor in eliciting the production of secondary metabolites in plant cells [Bose et al. 2013]. However, in contrast to this, previous studies have shown that exogenous application of gibberellic acid on peppermint plants led to lower menthol content in treated plants. The transcript levels of genes involved in the first steps of menthol biosynthesis, such as *gds*, *lh*, *ls* were not affected by gibberellic acid treatment, but the expression of genes involved in later stages, such as *neo-red* and *m-deh* was down-regulated by applying this treatment [Soleymani et al. 2015]. Gibberellic acid treatments increase the total phenolic compound of peppermint (especially caffeic acid) by enhancing PAL enzyme activity. Moreover, the positive effects of gibberellic acid treatments on the formation of trichomes and increasing their density and diameter, leading to higher essential oil content was reported in *Menta arvensis* plants [Bose et al. 2013], and similar effects could be found in peppermint through further research.

Melatonin. Melatonin is a brassinosteroid hormone that plays several important roles in regulating different physiological processes such as germination, rooting and defensive responses against plant stresses [Haydari et al. 2019]. According to previous studies, using melatonin as a plant growth regulator during peppermint cultivation can significantly increase its essential oil yield [Haydari et al. 2019]. Haydari et al. [2019] reported that treatment of peppermint with melatonin at a concentration of 10–30 M (40 d after seed sowing) increased total concentrations of oxygenated monoterpenes and oxygenated sesquiterpenes. The amount of monoterpene hydrocarbons was not affected by mel-

atonin treatments, but the foliar application of melatonin at a dose of 30 M increased menthol content by 14.3%. In addition, the amount of menthone and pulegone in treated plants was higher than that of the control, and menthofuran decreased with increasing melatonin treatment concentration from 10 to 30 M. To date, the mechanisms of melatonin effect on changes in enzymatic activities, biological pathways and gene expression in peppermint have not been investigated.

CONCLUSION

Given the economic importance of plant secondary metabolites and the increase in their demand, researchers are looking for inexpensive and useful strategies to increase the content and quality of plant secondary metabolites. Using abiotic elicitors such as UV radiation, salt and drought stress, and phytohormones during different growth stages of peppermint could be introduced as an effective method to enhance the content and quality of desired compounds. The effect of abiotic elicitors on secondary metabolites of peppermint is often correlated with the dosage, concentration or severity of abiotic elicitors and choosing the appropriate dosage or intensity is remarkably important in achieving the desired results. Abiotic elicitors can affect the secondary metabolites profile of peppermint mostly through changes in gene expression and plant enzymatic activity. In recent decades, most of the conducted research has covered the effects of abiotic elicitors on essential oils and monoterpenes (especially menthol) in peppermint, and more research on other important secondary metabolites of peppermint is required.

ACKNOWLEDGEMENTS

Not applicable.

AUTHORS' CONTRIBUTION STATEMENT

ANK, GE and MER compiled the literature and wrote the manuscript. MG edited and reviewed the manuscript. All authors approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

SOURCE OF FUNDING

This research received no external funding.

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