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REVIEW

# Traditional *in vitro* strategies for sustainable production of bioactive compounds and manipulation of metabolomic profile in medicinal, aromatic and ornamental plants

**Mohsen Niazian**[1] · **Paolo Sabbatini**[2]

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## Abstract

***Main conclusion*** **Precursor feeding, elicitation and culture medium parameters are traditional in vitro strategies to enhance bioactive compounds of medicinal, aromatic, and ornamental plants (MAOPs). Machine learning can help researchers find the best combination of these strategies to increase the secondary metabolites content of MAOPs.**

**Abstract** Many requirements for human life, from food, pharmaceuticals and cosmetics to clothes, fuel and building materials depend on plant-derived natural products. Essential oils, methanolic and ethanolic extracts of in vitro undifferentiated callus and organogenic cultures of medicinal, aromatic, and ornamental plants (MAOPs) contain bioactive compounds that have several applications for various industries, including food and pharmaceutical. In vitro culture systems provide opportunities to manipulate the metabolomic profile of MAOPs. Precursors feeding, elicitation and culture media optimization are the traditional strategies to enhance in vitro accumulation of favorable bioactive compounds. The stimulation of plant defense mechanisms through biotic and abiotic elicitors is a simple way to increase the production of secondary metabolites in different in vitro culture systems. Different elicitors have been applied to stimulate defense machinery and change the metabolomic profile of MAOPs in in vitro cultures. Plant growth regulators (PGRs), stress hormones, chitosan, microbial extracts and physical stresses are the most applied elicitors in this regard. Many other chemical tolerance-enhancer additives, such as melatonin and proline, have been applied along with stress response-inducing elicitors. The use of stress-inducing materials such as PEG and NaCl activates stress tolerance elicitors with the potential of increasing secondary metabolites content of MAOPs. The present study reviewed the state-of-the-art traditional in vitro strategies to manipulate bioactive compounds of MAOPs. The objective is to provide insights to researchers involved in in vitro production of plant-derived natural compounds. The present review provided a wide range of traditional strategies to increase the accumulation of valuable bioactive compounds of MAOPs in different in vitro systems. Traditional strategies are faster, simpler, and cost-effective than other biotechnology-based breeding methods such as genetic transformation, genome editing, metabolic pathways engineering, and synthetic biology. The integrate application of precursors and elicitors along with culture media optimization and the interpretation of their interactions through machine learning algorithms could provide an excellent opportunity for large-scale in vitro production of pharmaceutical bioactive compounds.

**Keywords** Antioxidants · Degree of differentiation · Inductive stresses · Precursors · Secondary metabolites · Tolerance-enhancer elicitors

## Abbreviations

| | |
|---|---|
| ANN | Artificial neural network |
| ABA | Abscisic acid |
| CKXs | Cytokinin oxidase/dehydrogenase enzymes |
| JA | Jasmonic acid |
| KAR1 | Karrikinolide |
| LED | Light-emitting diodes |
| MAOPs | Medicinal, aromatic and ornamental plants |
| MDA | Malondialdehyde |
| MJ | Methyl jasmonate |
| PEG | Polyethylene glycol |
| PGRs | Plant growth regulators |

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✉ Mohsen Niazian
mniazian@ut.ac.ir

Extended author information available on the last page of the article

| | |
|---|---|
| RSM | Response surface methodology |
| SA | Salicylic acid |
| SW | Smoke water |
| UV | Ultraviolet |
| YE | Yeast extract |

## Introduction

Medicinal, aromatic and ornamental plants (MAOPs) carry bioactive compounds that have several industrial applications such as food, flavoring, pharmaceutics, cosmetics and fragrances, and agrochemical. These compounds, especially secondary metabolites, are particularly valuable for the pharmaceutical industry: they are (i) effective in modulating the metabolic processes of the human body and treating diseases in a multi-target spectrum manner, and they are (ii) popular because safety and reduced side effects (Niazian et al. 2019a; Marchev et al. 2020). The above-mentioned reasons lead to an increased demand for natural bioactive compounds. However, in comparison to major food crops, plant breeders are less focused in improving medicinal and aromatic plants (Niazian 2019). Nevertheless, the steady increase in human population and the new challenges imposed by global warming are the factors that limited natural resources available to fulfil the growing demands of natural bioactive compounds. In this regard, plant in vitro system is one of the biotechnology-based breeding methods (BBBMs) with the potential to create alternative, sustainable and cost-effective production of bioactive substances. In vitro culture systems are independent from environmental, geographical and seasonal limitations (Chandran et al. 2020), as well as the plant developmental stage (Li et al. 2020). In vitro culture systems are able to produce plants with different chemical profile from the intact plants. This has been reported in *Ziziphora persica* (Zare-Hassani et al. 2019) and *Echinacea purpurea* (L.) Moench (Lema-Rumińska et al. 2019). Cabañas-García et al. (2021) reported nine new metabolites in methanolic extracts of callus culture of *Coryphantha macromeris,* not reported in greenhouse-grown plants. Different culture systems, including callus, shoot, roots, hairy roots and suspension cell cultures can be used for in vitro production of bioactive compounds of MAOPs. Finding the best in vitro culture system is very important to reach commercial scale production of targeted bioactive compound(s). For example, in white leadwort (*Plumbago zeylanica* L.), a 6.5-fold increase in plumbagin production was reported in semi-solid root-obtained callus culture (Sharma and Agrawal 2018), whereas liquid root suspension culture led to threefold increase in plumbagin content (Roy and Bharadvaja 2019). Therefore, setting up an in vitro culture system is a laborious and pivotal research task.

There are some traditional strategies to increase the production of bioactive compounds and modify the chemical profile of in vitro grown cell–tissue–organs. Manipulation of culture medium parameters (culture media optimization), including basal medium components, PGRs, carbon sources, additives and pH, can be considered from this perspective. Application of peptone (2 g/L) as additive in B5 medium, along with 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/L), led to 16.3-fold, 12.39-fold and 5.03-fold increased production of podophyllotoxin, kaempferol and quercetin in callus culture of *Dysosma pleiantha* (Hance) Woodson, (Karuppaiya and Tsay 2020). Different combinations and concentrations of 2,4-D and benzyladenine (BA) led to different contents of phenols and flavonoids bioactive compounds in callus culture of *Cnidium officinale* (Adil et al. 2018). Several concentrations (0.0, 0.2, 1.0 and 2.0 mg/L) of auxin PGRs, including 2,4-D, picloram, and α-naphthalene acetic acid (NAA) in combination with different concentrations (0.0, 0.1, 0.5 and 1.0 mg/L) of kinetin, 6-benzyladenine (BA), and thidiazuron (TDZ) cytokinins were evaluated in callus culture of *Sophora flavescens*. Research reported a tenfold increase of maackiain (Maac) bioactive compound in callus obtained in MS medium supplemented with 2.0 mg/L picloram + 0.5 mg/L BA than that of the field-grown plant root (Park et al. 2020a). These results indicate the importance of optimizing the combinations and concentrations of PGRs in in vitro production of bioactive compounds of MAOPs.

Secondary metabolites, as one of the most important and valuable chemical compounds synthesized by plants, are responsible for regulating plant interaction with the environment. These chemical defense molecules are secreted when plants are under biotic and abiotic stress and support the survival of their carrier plants under stressful conditions (Giri and Zaheer 2016; Alvarado et al. 2019). Stimulation of the plant defense machine is an important strategy to increase the production of specific defense molecules, including antioxidant secondary metabolites. By adding chemicals to culture medium and/or manipulation of artificially maintained in vitro growths through physical stresses, researchers are able to change metabolomic profile of plants. Adding sodium nitroprusside, as carrier of nitric oxide (NO), affected both non-enzymatic and enzymatic antioxidative systems in callus culture of *Ficus religiosa* (Hesami et al. 2020b).

Through precursor feeding, elicitation and manipulation of culture medium parameters, it was observed a different accumulation of bioactive compounds in solid and liquid cell, callus and organogenic cultures of MAOPs. In general, traditional in vitro strategies to manipulate metabolomic profile of different MAOPs are simpler than other complex BBBMs such as metabolic pathways engineering and targeted genome-editing methods.

## The effect of organogenesis on synthesis of valuable bioactive compounds

Callus cultures have great potential for sustainable production of bioactive compounds of endangered species of MAOPs (Koufan et al. 2020). However, the degree of differentiation (organogenesis) is an important factor that affects the in vitro production of secondary metabolites. In relation to different plant species, higher amount of metabolite production may be obtained from regenerated organs (shoot–root–bulblets) and/or undifferentiated callus cultures. In general, undifferentiated calluses are not efficient as shoots and bulblets for secondary metabolite production (Santos et al. 2020). Among differentiated organ cultures, hairy root culture has high productivity over other culture types (Chandran et al. 2020). In *Schisandra henryi* C. B. Clarke, a medicinal plant endemic to China, the comparative analysis of in vitro solid microshoot and callus cultures with plant material grown in vivo, revealed that higher amounts of dibenzocyclooctadiene lignans, phenolic acids, and flavonoids secondary metabolites were obtained in the extracts from micro-shoot cultures than from callus cultures and intact plants. In *Phellodendron chinense*, in vitro regenerated plantlets produced higher contents of berberine and phellodendrine than callus cultures (He et al. 2020). These results may be related to the different expression levels of genes involved in the biosynthesis pathways of secondary metabolites in callus and regenerated organs. In addition to differentiation degree, type of culture system should also be considered to enhance the accumulation of valuable bioactive compounds. Different in vitro shoot culture systems, including solid media and two liquid cultures (stationary and agitated), were compared in *Eryngium alpinum* (L.) and solid shoot culture was assigned as the best in vitro system for improved accumulation of phenolic acids and flavonoids (Kikowska et al. 2020). Plant cell culture is the most effective liquid culture system for the production of bioactive compounds and the only one used at an industrial level so far. An increase in silymarin content has been reported in cell suspension culture of *Silybum marianum* following to gamma rays elicitation (El-Garhy et al. 2021).

## Precursor feeding

Adding intermediate compounds, involved in the biosynthetic pathway of a specific bioactive compound, is a strategy to increase the accumulation of the final product (Yue et al. 2016). In clover (*Trifolium resupinatum*), feeding glutathione to callus cultures led to increased contents of phenols, flavonoids and antioxidant activities (Munim Twaij et al. 2019). Precursor feeding can also enhance the accumulation of secondary metabolites in in vitro cultures of MAOPs. Precursors can be added to culture medium as chemical additives and some of them may also induce stress to in vitro cultures (Qu et al. 2011). Different concentrations of sodium acetate (1, 5, 25, 50, 100 and 200 mg/L) and L-tyrosine (1, 5, 25, 50, 100 and 200 mg/L), as precursors of plumbagin biosynthetic pathway, were applied in callus cultures of chitrak (*Plumbago zeylanica* L.). Authors reported 2.07 and 2.64-fold increase in accumulation of plumbagin using 1 mg/L sodium acetate and 25 mg/L L-tyrosine, respectively (Singh et al. 2020b). Boonsnongcheep et al (2019), evaluated the effects of sodium acetate (10, 50 and 100 mg/L) and L-alanine (0.1, 1.0 and 5.0 mM) as precursors of plumbagin in in vitro cultures of *Drosera burmannii* and *Drosera indica*. Authors reported that precursor feeding with sodium acetate (50 mg/L for 3 days) had positive effect (1.2-fold) on the accumulation of plumbagin in the aerial part of *D. indica*. Mirmazloum et al. (2019) added three precursors of the salidroside biosynthesis pathway (tyramine, 4-hydroxyphenylpyruvate and tyrosol) in callus cultures of roseroot (*Rhodiola rosea* L.) and reported a significant increase (26-fold higher than the control) of salidroside in media with tyrosol precursor feeding (2 mM for 96 h). This study also reported that precursor feeding led to the significant increase in the expression of the UDP-glycosyltransferase gene (Mirmazloum et al. 2019). TRPH, TRPY and LOG were used as precursors of acetylechitamine in the callus culture of *Alstonia scholaris* (L.) and TRPH (50 mg/L in 10 days incubation) was determined as the most efficient precursor for the enrichment of acetylechitamine, which led to a 10.7-fold increase when compared to the control (Jeet et al. 2020). Feeding 4′-O-methylnorbelladine, as precursor of amaryllidaceae alkaloids, led to the significant increase of galanthamine and lycorine accumulation in in vitro bulblets of *Narcissus tazetta* (Tarakemeh et al. 2019). All mentioned examples show that finding the best type and concentration of precursors, involved in the biosynthetic pathway of secondary metabolites, is important to increase in vitro production of targeted compounds. This requires a lot of trial and error experiments.

## Elicitation

Elicitor refers to compounds able to stimulate the plant defense systems (Alvarado et al. 2019), and when applied in small quantity have the potential to increase the production of compounds pivotal for plant survival under stressful conditions. By this definition, elicitation refers to enhanced

synthesis of secondary metabolites (Thakur et al. 2019). Elicitors can affect plant physiological and biochemical traits through binding to receptors on the plasma membrane and trigger the signal cascade. Elicitors may induce secondary metabolites production through the activation of the intracellular transduction system, NADPH cascade, production of reactive oxygen species, expression of defense related genes, GTP binding proteins, high intracellular cAMP and $Ca^{2+}$ and other secondary messengers with mitogen-activated protein kinases (Singh et al. 2020b). Biotic and abiotic elicitors can be used individually and/or in combination together. There is wide range of elicitors that increase the accumulation of valuable secondary metabolites by stimulating stress responses in plants. However, some of these elicitors are stress tolerance-enhancer molecules, which will be discussed in the following sections. Examples of some recently applied elicitors in different MAOPs are presented in Table 1. It can help researchers to find appropriate type and concentrations of elicitor(s) to target their bioactive compound(s) of interest. The classification and different types of these elicitors are presented in the following sections.

## Additive biotic elicitors

Stress triggering and stress tolerance-inducing chemicals can be added to culture media as biotic elicitors to enhance protection and the accumulation of both enzymatic and non-enzymatic antioxidants including secondary metabolites like phenolics, flavonoids, and caffeic acid derivatives (Santisree et al. 2020), and other bioactive compounds involved in defense responses. Some of the widely applied chemical elicitors of this category have been discussed in the following sections.

### PGRs

PGRs are the most well-known biotic elicitors in the in vitro culture system that can affect the accumulation of bioactive compounds, in addition to their pivotal effect on propagation efficiency. It is well documented that different combinations and concentrations of PGRs can change the chemical profile of regenerants. Auxin PGRs can directly affect the degree of cellular differentiation and subsequently bioactive compounds production and metabolomic profile of regenerated plants (Santos et al. 2020). However, it should be considered that the type and concentration of auxins can lead to different results in terms of regeneration rate and metabolomic profile. Therefore, optimization of auxin PGRs is a crucial step in in vitro production of plant bioactive compounds. In *Narcissus tazetta*, it has been reported that the alkaloid profile of in vitro cultures varied with the type of applied auxins (2,4-D, picloram and NAA) and their concentrations. In addition, two new components (demethylmaritidine and tazettine) were found in differentiated tissue cultures on the medium containing NAA (25 or 50 µM) or in calli initiated with picloram (50 µM) (Tarakemeh et al. 2019). In callus culture of *Cnidium officinale* Makino, the production of phthalide and 3-butylidenephthalide compounds was significantly increased by the application of 2,4-D (Adil et al. 2018).

Cytokines are another important group of PGRs and their effects on metabolomics profile of in vitro cultured MAOPs have been extensively reported. Application of 6-benzyloaminopurine (BAP) (5 mg/L) led to the significant accumulation of saponin in in vitro microrhizomes of *Zingiber montanum* (Rajkumari and Sanatombi 2020). 2-isopentenyladenine (2iP), zeatin (Z) and BAP were studied in in vitro shoot regeneration of fireweed (*Chamerion angustifolium* L.) and highest multiplication rate was obtained by application of 2ip (1 mg/L) in MS medium. Authors reported that the highest contents of oenothein B (3.05 g/100 g DW), caffeic acid, gallic acid, ellagic acid and rosmarinic acid were reported in MS medium supplemented with 0.5 mg/L 2ip (Dreger et al. 2020). Cytokinin degradation by cytokinin oxidase/dehydrogenase enzymes (CKXs) is an important factor that should be considered in in vitro regeneration studies. The affinity of cytokinins to CKXs can affect their influence on micropropagation rate and metabolomic profile of regenerants. All the mentioned examples show the important roles of auxin and cytokinin PGRs in in vitro production of bioactive compounds of MAPOs. Many trials and errors are required to determine the optimal type and concentration of these PGRs and their combinations to reach to commercial scale production of targeted bioactive compounds.

### Stress hormones

Stress hormones are hormone signals involved in plant growth, development, photosynthesis, and defense mechanisms against biotic and abiotic stresses (Ahmadi et al. 2014). Abscisic acid (ABA), jasmonic acid (JA), MJ, and salicylic acid (SA) are the most commonly used signaling compounds that have been applied as biotic elicitors for elicitation of in vitro cultures of different MAOPs (Thakur et al. 2019). These stress hormones are involved in many signal transduction pathways and along with other PGRs can preserve and keep the integrity of plant cells under stressful conditions through up-regulating the antioxidant defense mechanisms (Raza et al. 2020). Exogenous application of JA and MJ in in vitro cultures can increase the accumulation of reactive oxygen species (ROS), activate antioxidant enzymes and expression of defense-related genes, and subsequently increase the accumulation of secondary metabolites (Ho et al. 2020). In anther-derived callus cultures of caper (*Capparis spinosa* L.), the effect of different concentrations of salicylic acid (50, 100 and 150 mg/L) and

**Table 1** Biotic and abiotic elicitors applied in in vitro culture systems to modify the accumulation of bioactive compounds in medicinal, aromatic and ornamental plants

| Plant species | Culture system | Basal culture medium | Bioactive compound(s) of interest | Changed amount of targeted bioactive compound (±fold) | Applied elicitor to target metabolomic profile | Reference |
|---|---|---|---|---|---|---|
| *Ajuga bracteosa* | Liquid adventitious roots | MS | Phenolic and flavonoids content | – | Light spectra | Ali et al. (2019b) |
| *Alstonia scholaris* | Solid callus | MS | Acetylechitamine | + 15.0 | CHT, MJ, SA, KCL, NaCl, PEG | Jeet et al. (2020) |
| *Aster scaber* | Liquid hairy root | MS | Phenols and flavonoids | – | MJ, YE | Ghimire et al. (2019) |
| *Astragalus membranaceus* | Liquid hairy root | SH | Astragaloside | – | YE | Park et al. (2020b) |
| | Liquid hairy root | – | Isofavones (formononetin and calycosin) | + 12.45 and + 6.17% | CHT | Gai et al. (2019) |
| *Bacopa monnieri* | Liquid cell suspension | B5 | Bacoside | – | JA, SA | Koul and Mallubhotla (2020) |
| *Calendula officinalis* | Liquid hairy root | MS | Triterpenoids (oleanolic acid saponins) | + 20% | CHT, JA | Alsoufi et al. (2019) |
| Caper (*Capparis spinosa*) | Solid anther-derived calli | B5 | Rutin flavonoid (vitamin P) | + 2.22–2.44 | SA, MJ | Kianersi et al. (2020) |
| Chitrak (*Plumbago zeylanica* L.) | Solid callus | MS | Plumbagin | + 12.08 | CHT, YE, proline, lysine, salicylic acid, $CdCl_2$, $Pb(NO_3)_2$ | Singh et al. (2020b) |
| *Corylus avellana* | Liquid cell suspension | MS | Paclitaxel | – | Fungal elicitation (*Camarosporomyces flavigenus*) | Salehi et al. (2020b) |
| Danshen (*Salvia miltiorrhiza*) | Liquid hairy root | B5 | Tanshinone | + 3.5 | MJ, YE, $AgNO_3$ | Wei et al. (2020) |
| Date palm *(Phoenix dactylifera* L.) | Liquid cell suspension | MS | Phenols (catechin, caffeic acid, kaempferol) | – | YE, SA, $CdCl_2$, $AgNO_3$ | Al-Khayri and Naik (2020) |
| *Dendrobium Enopi* × *Dendrobium* Pink Lady | Solid protocorm-like-body | MS | Phenols and flavonoids | – | Light spectra (cool white light, far red, green, blue, red and blue-red LED), amino acids (L-leucine, glycine and proline) | Yeow et al. (2020) |
| *Dracocephalum kotschyi* | Liquid hairy root | MS | Phenols and flavonoids | – | Fe NPs | Nourozi et al. (2019) |
| *Drosera burmannii* and *Drosera indica* | Solid shoot | MS | Plumbagin | + 1.9–2.8 | Light spectra (white, blue, red LED) | Boonsnongcheep et al. (2019) |
| *Droseraceae peltata* and *Droseraceae muscipula* | Solid shoot | MS | Phenols | – | Light spectra | Makowski et al. (2019) |
| *Dysosma pleiantha* | Solid callus | B5 | Podophyllotoxin, kaempferol, quercetin | + 16.0, + 12.39, + 5.03 | Peptone, CH, Coconut water | Karuppaiya and Tsay (2020) |
| *Dysphania ambrosioides* L | Solid shoot | MS | Monoterpene ascaridole | – | CHT, SA, light spectra | de Carvalho et al. (2020) |
| False daisy (*Eclipta alba* L.) | Solid callus | MS | Phenols and flavonoids | – | Light spectra | Khurshid et al. (2020) |
| Feverfew (*Tanacetum parthenium*) | Liquid hairy root | MS | Parthenolide | – | YE, MJ, $AgNO_3$ | Pourianezhad et al. (2019) |

**Table 1** (continued)

| Plant species | Culture system | Basal culture medium | Bioactive compound(s) of interest | Changed amount of targeted bioactive compound (±fold) | Applied elicitor to target metabolomic profile | Reference |
|---|---|---|---|---|---|---|
| Fireweed (*Chamerion angustifolium* L.) | Solid shoot | MS | Oenothein B and phenolic acid | – | PGRs (2iP, Zeatin) | Dreger et al. (2020) |
| Flax (*Linum usitatissimum* L.) | Liquid suspension cell | MS | Phenol and lignans | – | ZnO, $TiO_2$ | Karimzadeh et al. (2019) |
| Garden cress (*Lepidium sativum* L.) | Solid callus | MS | Antidiabetic metabolites (α-glucosidase and α-amylase) | – | UV-C, melatonin | Ullah et al. (2019) |
| Garlic (*Allium sativum*) | Solid callus, somatic embryos, leaves and roots | MS | Alliin | – | $CdCl_2$ | Malik et al. (2020) |
| *Gloriosa superba* L | Liquid suspension cell | MS | Colchicine | + 8.0 | SA, YE, CH, $AgNO_3$ | Mahendran et al. (2018) |
| Gotu Kola (*Centella asiatica* L.) | Solid shoot | MS | Asiaticoside, *p*-coumaric acid, kaempherol | + 5.6, + 122, + 22.4 | MJ, ethephon, L-phenylalanine | Skrzypczak-Pietraszek et al. (2019) |
| *Helicteres isora* (L.) | Liquid suspension cell | MS | Diosgenin | + 9.1 | Microbial elicitors (*Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus niger*) | Shaikh et al. (2020) |
| *Hybanthus enneaspermus* (L.) | Liquid adventitious root | MS | L-Dopa | + 6.19 | SA, MJ, YE, $AgNO_3$ | Sathish et al. (2020) |
| *Hypericum aviculariifolium* and *Hypericum pruinatum* | Solid shoot | MS | Rutin flavonoid (vitamin P) | – | JA | Cirak et al. (2020) |
| Lavender (*Lavandula angustifolia* Mill) | Solid shoot | MS | Polyphenol | – | JA, SA | Miclea et al. (2020) |
| *Lippia alba* L | Solid shoot | MS | Linalool and germacrene | + % and − % | PEG | de Castro et al. (2020) |
| *Morus alba* (L.) | Liquid roots | MS | Mulberroside A, oxyresveratrol, resveratrol | – | YE, MJ | Inyai et al. (2020) |
| *Narcissus papyraceus* and-*Narcissus tazetta* | Solid shoot and callus | MS | Amaryllidaceae alkaloids | – | PGRs (2,4-D, picloram, NAA) | Tarakemeh et al. (2019) |
| *Ocimum tenuiflorum* (L.) | Liquid hairy roots | MS | Ursolic acid, eugenol | + 5.6, + 6.0 | YE, MJ, SA | Sharan et al. (2019) |
| *Panax ginseng* | Liquid adventitious root | MS | Ginsenosides | + 2.59 | Fungal elicitor (*Fusarium oxysporum*, *Penicillium* sp. YJM-2013) | Wang et al. (2020) |
| *Piper cumanense* | Liquid cell suspension | MS | 5-hydroxymethylfurfural, phenol, (Z)-9-octadecenamide | – | MJ, SA | Rodríguez-Sánchez et al. (2020) |
| *Plumbago zeylanica* | Liquid root suspension | MS | Plumbagin | + 3.0 | YE, ME, MJ, SA | Roy and Bharadvaja (2019) |
| | Semi-solid callus | MS | Plumbagin | + 6.5 | YE, SA | Sharma and Agrawal (2018) |
| Rosemary (*Rosmarinus officinalis* L.) | Solid callus | MS | Rosmarinic acid | – | Melatonin | Coskun et al. (2019) |

**Table 1** (continued)

| Plant species | Culture system | Basal culture medium | Bioactive compound(s) of interest | Changed amount of targeted bioactive compound (±fold) | Applied elicitor to target metabolomic profile | Reference |
|---|---|---|---|---|---|---|
| Sacchi boti (*Fagonia indica*) | Solid callus | MS | Phenols and flavonoids | – | CHT, SA | Khan et al. (2019) |
| *Salvia leriifolia* | Solid callus and shoot | MS | Rosmarinic acid, caffeic acid, and salvianolic acid B | – | PEG | Hosseini et al. (2020) |
| *Salvia miltiorrhiza* | Liquid hairy roots | – | Salvianolic acid B | – | $KAR_1$, JA | Sun et al. (2020) |
| | Liquid hairy roots | – | Salvianolic and rosmarinic acids | – | Smoke water, $KAR_1$ | Zhou et al. (2018) |
| | Solid callus and liquid suspension cell | MS | Shikimic acid, tanshinol, protocatechuic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone, cryptotanshinone, tanshinone I, tanshinone II A | – | NaCl, $AgNO_3$, SA | Yu et al. (2019) |
| *Satureja khuzistanica* | Liquid nodal segments | MS | Rosmarinic acid | +2.43 | MWCNTs, MJ | Fatemi et al. (2020) |
| *Schisandra henryi* | Solid microshoot and callus | MS | Dibenzocyclooctadiene lignans and phenolic acids and flavonoids | +13 and +7 & +1.4 | PGRs (BA, IBA, $GA_3$) | Jafernik et al. (2020) |
| *Scutellaria bornmuelleri* | Liquid hairy roots | MS | Flavonoids (chrysin, wogonin, baicalein) | +9.15, +10.56, +13.25 | MJ, CHT, methyl-b-cyclodextrin | Gharari et al. (2020) |
| *Stevia rebaudiana* | Solid shoot | MS | Steviol glycosides | +60 | MJ, CHT, YE | Rasouli et al. (2021) |
| | Solid shoot | MS | Steviol glycosides (rebaudioside A and stevioside) | – | PEG | Ahmad et al. (2020) |
| Sweet basil (*Ocimum basilicum* L.) | Liquid suspension cell | MS | Chicoric acid, rosmarinic acid | +0.92, +1.25 | YE, $CdCl_2$, $AgNO_3$ | Açıkgöz (2020) |
| | Solid callus | MS | Rosmarinic acid | +5.0 | Melatonin | Duran et al. (2019) |
| *Teucrium polium* | Solid callus | MS | Phenol, flavonoids, antioxidants | – | MJA, SA | Hashemyan et al. (2020) |
| *Thymus lotocephalus* | Solid shoot | MS | Rosmarinic acid | – | YE, SA, $AgNO_3$ | Gonçalves et al. (2019) |
| *Thymus vulgaris*, *T. daensis*, *T. kotschyanus*, *Zataria multiflora* | Solid callus | MS | Thymol and carvacrol | – | ZnO | Mosavat et al. (2019) |
| *Trichosanthes cucumerina* (L.) | Liquid cell suspension | MS | Bryonolic acid | – | MJ, YE, CHT | Lertphadungkit et al. (2020) |
| Venus flytrap (*Dionaea muscipula* J. Ellis) | Liquid tissue | MS | Caffeic acid, ellagic acid, plumbagin | – | Bacterial elicitor (*Cronobacter sakazakii*) | Makowski et al. (2020) |

**Table 1** (continued)

| Plant species | Culture system | Basal culture medium | Bioactive compound(s) of interest | Changed amount of targeted bioactive compound (± fold) | Applied elicitor to target metabolomic profile | Reference |
|---|---|---|---|---|---|---|
| Winter cherry (*Withania somnifera* L.) | Solid callus | MS | Phenols and flavonoids | – | Light spectra | Adil et al. (2019) |
| *Zingiber montanum* | Liquid cell suspension | MS | Phenols and steroid | – | MJ, JA, SA | Rajkumari and Sanatombi (2020) |
| *Ziziphora persica* | Liquid shoot | MS | Alkan hydrocarbons | – | MJ, SA | Zare-Hassani et al. (2019) |

*CH* casein hydrolysate, *CHT* chitosan, *JA* jasmonic acid, $KAR_1$ karrikinolide, *LED* light-emitting diodes, *ME* malt extract, *NPs* nanoparticles, *MJ* methyl jasmonate, *MS* Murashige and Skoog's medium, *MWCNTs* multi-walled carbon nanotubes, *PEG* polyethylene glycol, *PGRs* plant growth regulators, *SA* salicylic acid, *SH* Schenk and Hildebrandt medium, *UV* ultraviolet, *YE* yeast extract

methyl jasmonate (1, 10 and 100 µM) elicitors on accumulation of rutin flavonoid (vitamin P) was assessed. Higher accumulation of rutin was obtained using 100 mg/L SA (2.22-fold) or 10 µM MJ (2.44-fold) when compared to the control (Kianersi et al. 2020). Elicitation of cell suspensions culture of *Piper cumanense* was conducted using methyl jasmonate and salicylic acid elicitors and effects of type, concentration (10 and 100 µM) and time of exposition (3, 12 and 24 h) of elicitors on production of metabolites were evaluated. Authors reported that highest contents of 5-hydroxymethylfurfural (6.3%), phenol (6.5%), and (Z)-9-octadecenamide (8.8%) were obtained by 100 µM of SA (Rodríguez-Sánchez et al. 2020). A comparison of jasmonic acid and salicylic acid elicitation with precursors (calcium pantothenate, cholesterol, sodium nitroprusside) on the stimulation of biomass and secondary metabolite production in cell suspension culture of *Bacopa monnieri* reported that highest bacoside content was obtained using salicylic acid at 1.0 mg/L (Koul and Mallubhotla 2020). In in vitro shoot culture of *Stevia rebaudiana*, effect of abiotic elicitor of MJ was compared with biotic elicitors of chitosan and yeast extract (YE). When compared with the control treatment, elicitors led to the higher production of steviol glycosides (SGs); however, the highest total SGs content ($23.83 \pm 0.25\%$) was obtained by MJ elicitation (100 mg/L) (Rasouli et al. 2021). Jasmonic acid elicitation changed secondary metabolite profile in in vitro shoot cultures of two endangered species from Turkish flora, *Hypericum aviculariifolium* and *H. pruinatum* and content of rutin secondary metabolite of JA treated plants was higher than control in vitro-regenerated and intact plants (Cirak et al. 2020). The effect of chitosan and jasmonic acid elicitors on triterpenoid production in hairy root cultures of *Calendula officinalis* was evaluated and the highest accumulation of oleanolic acid saponins (up to 20-fold) was obtained using JA elicitor (Alsoufi et al. 2019). Elicitation of in vitro shoot cultures of *Lavandula angustifolia* Mill with JA (0.5 mg/L) + SA (1.5 mg/L) led to the higher polyphenolic and chlorophyll content than the controls (Miclea et al. 2020). Skrzypczak-Pietraszek et al. (2019) investigated the effect of different combinations of ethephon (50 µM), methyl jasmonate (50 µM) and L-phenylalanine (2.4 g/L) on the accumulation of centellosides (asiaticoside and madecassoside), phenolic acids, and flavonoids secondary metabolites in in vitro shoot cultures of *Centella asiatica* (L.) Urban. In comparison with control cultures, concentrations of asiaticoside (up to 5.6-fold), *p*-coumaric acid (up to 122-fold) and kaempferol (up to 22.4-fold) were increased in MJA treated cultures. Hashemyan et al (2020) compared the effect of different concentrations of MJ (0, 50 and 100 µg/L) and SA elicitors on phytochemical profile of callus cultures of *Teucrium polium* (L.) and reported that the greatest contents of phenol, flavonoids and total antioxidant capacity were obtained in calli treated with 100 µg/L

SA. Different elicitors, including pectin, YE, salicylic acid (SA), cadmium chloride ($CdCl_2$), and silver nitrate ($AgNO_3$) have been applied in cell suspension culture of date palm (*Phoenix dactylifera* L.) and SA elicitor (50 mg/L) led to the highest accumulation of total phenolic content (catechin, caffeic acid, kaempferol) and radical scavenging activity, compared to the control (Al-Khayri and Naik 2020). Comparative analysis of different cell suspension cultures of *Zingiber montanum* elicited with MJ, JA and SA, each at 50, 100, 150 and 200 µM, showed that highest total phenolic and steroid contents were obtained from methyl jasmonate-treated cell cultures (Rajkumari and Sanatombi 2020). All the literatures reviewed here indicate the importance of finding the best type and concentration of stress hormone elicitor(s) to obtain the maximum accumulation of secondary metabolites of interest.

### Chitosan

Chitosan is one of the valuable polymeric deacetylated derivatives of chitin with biocompatibility, biodegradability, bioactivity and non-toxicity properties, which naturally found in shell of prawns or crabs and the cell wall of some fungi (Ahmadi and Shariatpanahi 2015). Chitosan has antimicrobial function and can suppress various bacterial and fungal species (Rashki et al. 2021). In addition to biotic stress, chitosan can enhance defense responses against abiotic stresses. In mung bean (*Vigna radiata*), both normal and nano-sized chitosan were applied under salt stress. Authors reported higher contents of phenol, flavonoids, proline and ascorbic acid and lower contents of malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$) of chitosan-treated seedlings in comparison with control seedlings under severe salinity stress (8 dS/m), which suggested the activation of the defense mechanisms by chitosan (Sen et al. 2020). In another study, Gerami et al. (2020) examined the effect of different concentrations of chitosan (0, 0.2, 0.4 and 0.6 g/L) on physiological and biochemical characteristics of stevia (*Stevia rebaudiana* Bertoni) under different levels of salinity stress (0, 50, 100 and 150 mM NaCl). They reported that chitosan led to increases in MDA, antioxidant enzymes activity (CAT and POX), and steviol glycosides contents of stevia seedlings under salt stress condition. Stress tolerance induced by chitosan application has also been reported in potato (*Solanum tuberosum* L.) (Muley et al. 2019). Chitosan has an important role in signal transduction pathways and by binding to transcription factors; it can affect the over-expression of defense genes and the biosynthesis of secondary metabolites (Singh et al. 2020b). Therefore, chitosan can be used as elicitor, both in vivo and in vitro, to enhance defense molecules and secondary metabolites of MAOPs under stressful condition. In basil (*Ocimum basilicum* L.) and lemon balm (*Melissa officinalis* L.), foliar spray of chitosan lactate led to increased accumulation of rosmarinic acid secondary metabolite (Hawrylak-Nowak et al. 2020). In another study, foliar spray of jasmonate (100, 250 and 500 µM), zinc oxide nanoparticles (ZnO-NPs) (20, 60 and 100 ppm) and chitosan (10, 50, and 100 µM) elicitors on carla (*Momordica charantia* L.) seedlings at the 4-leaf stage growth was evaluated. In comparison to a control treatment, all elicitors led to the significant increased amounts of secondary metabolites such as phenols, flavonoids, and carotenoids as well as carbohydrate and proline content. Among investigated elicitors, the highest phenolic content was obtained by applying 100 µM chitosan elicitor (Sharifi-Rad et al. 2020). Foliar spray of chitosan, jasmonic acid and salicylic acid elicitors led to the enhanced content of withanolide in *Withania somnifera* (L.) (Singh et al. 2020a).

Chitosan elicitation (100 mg/L for 24 h) led to 12.45- and 6.17-fold increases in the accumulation of formononetin and calycosin phytoalexins in hairy root cultures of *Astragalus membranaceus* (Gai et al. 2019). In recent in vitro studies, chitosan is mainly applied along with stress hormones in elicitation of different bioactive compounds. Khan et al. (2019), investigated effects of different concentrations of chitosan and salicylic acid elicitors in callus cultures of *Fagonia indica* and reported that both elicitors led to the higher accumulation of biomass, phytochemicals (phenolic and flavonoid), and antioxidant enzyme activities (peroxidase and superoxide dismutase), compared to the control treatment. However, maximum biomass, secondary metabolites contents, antioxidant enzyme activities and pharmacologically active components were obtained in chitosan treated cultures. In another study, chitosan was applied along with salicylic acid elicitor in in vitro culture of *Dysphania ambrosioides* and concomitant use of chitosan (50–100 mg/L) and salicylic acid (6–9 mg/L) led to the highest Z-ascaridole content (de Carvalho et al. 2020). Lertphadungkit et al. (2020) investigated the effect of methyl jasmonate, yeast extract, and chitosan on accumulation of bryonolic acid in cell suspension culture of *Trichosanthes cucumerina* (L.). Authors reported that chitosan (1 mg/mL for eight days) was superior over other applied elicitors in terms of produced bryonolic acid. The recent reviewed literatures showed co-application of chitosan with stress hormones elicitors. These reports suggest a complementary role for chitosan and stress hormones to reach higher amount of desired bioactive compounds of MAPOs.

### Microbial elicitors

Microbial extracts are an active group of elicitors that have been widely used for in vitro enrichment of valuable bioactive compounds by inducing plant resistance responses. Among them, fungal elicitors have been mentioned as one of the applicable strategies for in vitro enhancing of

secondary metabolites (Salehi et al. 2020a). Wang et al. (2020) applied different concentrations (0, 50, 100, 200, 400 mg/L) of pathogenic fungi *Fusarium oxysporum* and antagonistic fungi *Penicillium* sp. YJM-2013 in adventitious root culture of *Panax ginseng* for 5 days and reported that greatest ginsenosides accumulation was obtained by *Penicillium* sp. YJM-2013 at 200 mg/L. The antagonistic fungi enhanced the generation of signal molecules, activating the expression of transcription factors and functional genes and subsequently increased the accumulation of ginsenosides in adventitious root culture of *P. ginseng* (Wang et al. 2020). Fungal elicitation, using mixture of cell extract and culture filtrate derived from *Camarosporomyces flavigenus*, led to increased accumulation and secretion of paclitaxel in cell suspension culture of *Corylus avellana* (Salehi et al. 2020b).

Other types of microbial extracts, including bacterial extracts, have also been applied in in vitro studies. Microbial volatile organic compounds (mVOC), emitted by *Bacillus amyloliquefaciens* GB03 Rhizobacteria, led to the increased contents of menthone, menthol, pulegone, total phenolic compound, and antioxidant status in in vitro grown seedlings of peppermint (*Mentha piperita* L.) under salinity stress (Del Rosario Cappellari et al. 2020). Shaikh et al. (2020) compared the effects of bacterial and fungal elicitors, including *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus niger* in cell suspension cultures of *Helicteres isora* (L.) and reported that the greatest content of diosgenin (9.1-fold increase) was obtained using *E. coli* (1.5%). They also found that applied microbial elicitors led to the increased expression levels of 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) and cycloartenol synthase (*CAS*) genes, the regulatory genes in diosgenin biosynthetic pathway (Shaikh et al. 2020). Bacterial elicitation (*Cronobacter sakazakii* bacteria lysate) led to increased synthesis of myricetin, caffeic acid, ellagic acid and plumbagin in liquid tissue culture of Venus flytrap *(Dionaea muscipula* J. Ellis) (Makowski et al. 2020).

Yeast extract is another category of microbial elicitors that has been widely applied for in vitro enhancement of secondary metabolites. Yeast extract stimulate astragaloside accumulation, phenols and flavonoid contents in hairy root cultures of *Astragalus membranaceus* (Park et al. 2020b) and *Aster scaber* (Ghimire et al. 2019), tanshinone in transformed hairy roots of danshen (*Salvia miltiorrhiza*) (Wei et al. 2020), and ursolic acid and eugenol in hairy root cultures of *Ocimum tenuiflorum* (L.) (Sharan et al. 2019). The concomitant use of YE (2.5 mg/L) and MJ (100 µM) elicitors led to the enhanced accumulation of parthenolide and increased expression of *TpPTS* gene in hairy root culture of feverfew (*Tanacetum parthenium*) (Pourianezhad et al. 2019). Similar results were observed in liquid root cultures of *Morus alba* (L.) and the greatest amounts of mulberroside A, oxyresveratrol and resveratrol were obtained by mixture of YE (2 mg/ml) and MJ (200 µM) elicitors (Inyai et al. 2020). Singh et al. (2020b) reported a dramatic increase in plumbagin content (12.08–fold) in callus culture of chitrak using the integrative application of chitosan (50 mg/L) and YE (100 mg/L) elicitors. Yeast extract elicitation (150 mg/L) led to the threefold increase in plumbagin concentration in root suspension culture of *Plumbago zeylanica* (Roy and Bharadvaja 2019). The positive effect of YE on accumulation of colchicine and thiocolchicoside in cell suspension cultures of *Gloriosa superba* (Mahendran et al. 2018) and enrichment of schisandra and dibenzocyclooctadiene lignans in micro-shoot cultures of *Schisandra chinensis* (Szopa et al. 2018), has been reported. The comparative analysis of yeast extract elicitation with $CdCl_2$ and $AgNO_3$ elicitors in cell suspension cultures of sweet basil (*Ocimum basilicum* L.) showed the superiority of YE over the $CdCl_2$ and $AgNO_3$, as the greatest contents of chicoric acid and rosmarinic acid were obtained using 200 mg/L YE (Açıkgöz 2020). Gonçalves et al. (2019) also reported the superiority of YE over the SA and $AgNO_3$ elicitors in increasing of rosmarinic acid content in in vitro shoot culture of *Thymus lotocephalus*. The summary of reviewed researches in this section indicates that fungal, bacterial and YE are the most widely applied microbial elicitors to increase the bioactive compounds of MAOPs. The superiority of YE over the stress hormones has been reported in some studies. Trial and error experiments are required to find the best type and concentration of microbial elicitor to enhance the in vitro production of bioactive compound(s) of interest in targeted MAOPs.

#### Smoke water (SW) and karrikinolide ($KAR_1$)

Smoke-derived karrikinolide ($KAR_1$) and smoke water (SW)—generated by drawing smoke through water for one hour—are two plant growth promoting substances and responsible for eliciting plant seeds germination and growth responses in plant tissues. $KAR_1$ is a compound containing a butenolide fused to a pyran ring and has very high activity with plant cells, as its effectiveness is comparable to the one of plant hormones. SW and $KAR_1$ can also be used as elicitors to enhance accumulation of secondary metabolites (Kępczyński 2020). Smoke water and $KAR_1$ elicitation of 18-day-old hairy root cultures of *Salvia miltiorrhiza* led to the increased accumulation of salvianolic and rosmarinic acids compounds (Zhou et al. 2018). Sun et al. (Sun et al. 2020) reported that hairy root cultures of *Salvia miltiorrhiza* were respond to $KAR_1$ in the first step by bursting of calcium-calmodulin ($Ca^{2+}$-CaM), brassinolide (BL) and JA. $Ca^{2+}$ and BL act as upstream signaling molecules in the production of JA. Therefore, $KAR_1$- induced enhanced accumulation of salvianolic acid mediated by $Ca^{2+}$-CaM and BL and dependent on the generation of JA. Although $KAR_1$ and SW elicitation has been reported in hairy root cultures

of *S. miltiorrhiza*, however, their application in other in vitro culture systems of MAOPs and their effect on the accumulation of secondary metabolites should be investigated.

## Abiotic elicitors

Abiotic elicitors can be classified as chemical agents and physical treatments.

### Chemical elicitors

Chemical agents including heavy metal ions, hyperosmotic stress, signaling molecules and metal-based nanoparticles can be added to culture media as additive elicitors to affect synthesis and accumulation of valuable bioactive compounds and secondary metabolites. Most recently applied additive abiotic elicitors are presented in the following sections.

**Heavy metal ions** Heavy metals elicitation is one of the most applied strategies to enhance secondary metabolite content of MAOPs in different in vitro culture systems including cell, callus, hairy root, and organ cultures. Metal cadmium chloride ($CdCl_2$) applied in callus, somatic embryos, leaves and roots in vitro cultures of garlic (*Allium sativum*) and its positive effect on increasing alliin content was reported (Malik et al. 2020). In vivo root-obtained callus cultures of chitrak were subjected to different concentrations of $CdCl_2$ (1, 5, 25, 50, 100 and 200 mg/L) and $Pb(NO_3)_2$ (1, 5, 25, 50, 100 and 200 mg/L) heavy metals. Results reported 1.14- and 1.07-fold increases in amount of plumbagin when compared to the control, achieved through applying 5 mg/L $CdCl_2$ and 25 mg/L $Pb(NO_3)_2$, respectively (Singh et al. 2020b). Açıkgöz (2020) applied different concentrations of $CdCl_2$ and $AgNO_3$ in cell suspension culture of sweet basil and reported a positive effect of $AgNO_3$ on accumulation of linalool and estragole pharmaceutical compounds. $AgNO_3$ elicitation led to a 3.13-fold higher accumulation of L-dopa, than unelicited culture, in adventitious root cultures of *Hybanthus enneaspermus* (L.) F. Muell (Sathish et al. 2020).

**Artificial water deficiency and salinity stresses inducting elicitors** Exposing in vitro cultures to artificially induced abiotic stresses is a strategy to trigger metabolic adjustments and thus enhance the accumulation of secondary metabolites. Polyethylene glycol (PEG) and sodium chloride (NaCl) are the most applied additives to induce artificially drought and salinity stresses. These two combinations have been applied as abiotic elicitors to enhance secondary metabolite accumulation of different plant species.

NaCl elicitation (50 mmol/L) in cell suspension culture of *Salvia miltiorrhiza* Bunge led to the significant increase of content of secondary metabolites of shikimic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone II A (Yu et al. 2019). Drought stress imposed by PEG 6000 (4%) led to the significant increase in content of rebaudioside A and stevioside glycosides, total phenolic content, total flavonoid content, total antioxidant capacity, total reducing power and 1,1-diphenyl-2-picrylhydrazyl(DPPH)-free radical scavenging activity in shoot cultures of *Stevia rebaudiana* Bertoni (Ahmad et al. 2020). Hosseini et al. (2020) applied different concentrations of PEG 6000 (5, 7.5 and 10% w/v) in plantlets and calli cultures of *Salvia leriifolia* and observed increased accumulation of rosmarinic acid, caffeic acid, and salvianolic acid B secondary metabolites in both culture types subjected to PEG 5%.

**Abiotic nanoparticles-based elicitors** Nanoelicitors covers both biotic and abiotic elicitors; however, only the abiotic group has been reported here. Nanoparticles stress has great potential to stimulate the plant defense mechanisms and increase the accumulation of bioactive compounds in medicinal plants (Shahhoseini et al. 2020). In vivo nano-silicon (nano-Si) elicitation led to increased contents of glycine betaine (GB), favonols (quercetin and rutin), and enzymatic antioxidants and decreased amount of $H_2O_2$ and MDA in sugar beet (*Beta vulgaris* L.) under water stress (Namjoyan et al. 2020). There is an increasing trend in the application of nanoparticles as efficient non-biologic elicitors to improve in vitro biosynthesis of secondary metabolites. Mosavat et al. (2019) evaluated the effect of different concentrations of ZnO nanoparticles (ZnONPs) (100 and 150 mg/L) on growth and secondary metabolites (thymol and carvacrol) content of callus cultures of different aromatic *Thymus* species (*T. vulgaris*, *T. daensis* and *T. kotschyanus*) and *Zataria multiflora*. Authors reported that the greatest accumulations of thymol and carvacrol were achieved in *T. kotschyanus* and *T. daenesis* under stress of 150 mg/L of ZnO nanoparticles. Different concentrations of ZnO (0, 30, 60, and 120 mg/L) and $TiO_2$ (0, 50, 100, and 150 mg/L) nanoparticle elicitors have been applied in suspension cell culture of flax (*Linum usitatissimum* L.) and highest accumulations of total phenol and lignans were obtained using 30 and 60 mg/L ZnO (Karimzadeh et al. 2019). Multi-walled carbon nanotubes (MWCNT) elicitor led to a 2.43-fold increase of the content of rosmarinic acid in liquid nodal segment culture of *Satureja khuzistanica* (Fatemi et al. 2020). Nourozi et al. (2019) applied iron oxide nanoparticles (Fe NPs) in hairy root cultures of *Dracocephalum kotschyi* Boiss and reported that Fe NP increased the activity of antioxidant enzymes, total phenolic and flavonoid content and the highest rosmarinic acid content was obtained using 75 mg/L Fe NP in 24 h elicitation time exposure. Increased production of phe-

nolics, flavonoids, phenylalanine ammonialyase and antioxidant activity was reported in callus culture of *Caralluma tuberculata* using 90 µg/L silver nanoparticles (AgNPs) (Ali et al. 2019a). The summary of literatures reviewed in this section indicates that there are different types of abiotic nanoparticles-based elicitors to enhance the accumulation of bioactive compounds of MAOPs and mainly working through stimulate the plant defense mechanisms. Finding the best type of concentrations of these elicitors needs a lot of trial and error experiments.

### Physical abiotic elicitors in in vitro cultures to enhance production of bioactives

All the above-mentioned elicitors have been used to manipulate in vitro culture systems from the inside of the culture vessels and can be used as additives in culture media. However, there are also two physical treatments for manipulation of in vitro cultures from the outside of the culture vessels and can be considered as natural elicitors. Artificially maintained in vitro growth conditions can influence growth and morphogenetic responses of plant cell cultures. In vitro culture vessels can be exposed to physical treatments of high and low temperatures and light stresses to enhance production of important bioactive compounds.

Light is an important physical factor that many aspects of plant cell growth and development depend on it. Growth, biomass accumulation and production of metabolites in plants largely depend on the wavelength, photoperiod and flux density of the applied light (Ascencio-Cabral et al. 2008). By inducing photo-oxidative changes in plant cells, monochromatic light regimes can control the cell growth and production of metabolites (Samuolienė et al. 2016). Alteration in the number, content and profile of volatile constituents of *Achillea millefolium* with changes in the wavelength and intensity of light has been reported (Alvarenga et al. 2015). Depending of plant species and genotype, different light spectra can lead to different results in terms of content of in vitro-produced bioactive compounds. In *Lippia gracilis*, higher production of carvacrol was obtained by blue light (Lazzarini et al. 2018), whereas the highest carvacrol content in *Plectranthus amboinicus* was obtained from monochromatic red light (Silva et al. 2017). Enhanced plumbagin accumulation has been reported in in vitro shoot cultures of *Drosera burmannii* (2.8 fold) and *Drosera indica* (1.9 fold) under blue LED light (Boonsnongcheep et al. 2019). In in vitro adventitious roots culture of *Ajuga bracteosa*, effect of different spectrums of light on the growth, secondary metabolism and biosynthesis of phenolic acids was assessed and significant variations in the levels of important phenolic acids, such as gallic acid, catechin, rutin, caffeic acid, myricetin and apigenin, with the lights of different spectra has been reported. Maximum superoxide dismutase and peroxidase activities were observed from blue spectral light (Ali et al. 2019b). In another study, effects of light spectra, including blue (B), red (R), white and combinations of B:R (1:1, 2:1, 1:2), from light-emitting diodes (LEDs) and fluorescent lamps, were evaluated on *in vitro* cultures of *Dysphania ambrosioides* L and revealed that the blue monochromatic LEDs led to the highest content of *p*-Cymene and had negative effect on Z-ascaridole. The white fluorescent light promoted a greater conversion of α-terpinene into ascaridole (de Carvalho et al. 2020). The in vitro callus cultures of *Withania somnifera*, an endangered medicinal plant, were subjected to different monochromatic light wavelengths (violet, blue, green, yellow and red) and red light was assigned as optimum light spectra that led to the maximum biomass accumulation and antioxidant activity of callus cultures (Adil et al. 2019). Authors also reported the positive effect of violet light on the phenols and flavonoids synthesis in callus cultures that indicates to it that this light spectrum has activated defense mechanisms of *W. somnifera*. Khurshid et al. (2020) investigated the effects of multispectral lights, including yellow, green, white, blue and red, on accumulation of antioxidant and antidiabetic secondary metabolites in callus culture of *Eclipta alba* and reported that the highest phenolics and flavonoids contents along with highest accumulation of coumarin, eclalbatin, wedelolactone, demethylwedelolactone, β-amyrin and luteolin were obtained under red light. In vitro shoot cultures of *Droseraceae peltata* and *Droseraceae muscipula* were subjected to two light regimes, including fluorescent light of 120 µmol $m^{-2}$ $s^{-1}$PPFD as control and changed spectral composition—blue–red (ratio 6:1) LED light of 120 µmol $m^{-2}$ $s^{-1}$ PPFD. Authors reported that blue–red LED increased proline level of *D. peltata* and decreased POD activity in both species. Authors mentioned that blue–red light regime was not an effective elicitor of phenolic compounds in both studied plants species (Makowski et al. 2019).

Ultraviolet (UV) irradiation has been reported as one of the major inducers of secondary metabolites in plants. Plant secondary metabolites have UV-B screening effects that can quench the reactive oxygen and nitrogen species created under UV-B radiation. This property has made secondary metabolites popular for sunscreens and cosmetics, pharma and nutraceuticals (Takshak and Agrawal 2019). UV-B and UV-C irradiations can affect accumulation and composition of secondary metabolites of plants (Santisree et al. 2020). In callus culture of *Lepidium sativum*, effects of different concentrations of melatonin (0, 5, 10, 15, 20, 25 and 50 µM) and UV-C exposure durations (10, 30, 60, 90, 120 and 150 min) were assessed on biosynthesis of antioxidant and antidiabetic metabolites separately. Authors reported that melatonin, as an abiotic elicitor, at concentration of 20 µM, was more effective than UV-C treatment and led to the higher antidiabetic activities (*α*-glucosidase

and $\alpha$-amylase) in callus cultures (Ullah et al. 2019). The summary of reviewed researches in this section shows the importance of physical light elicitors in enhancing bioactive compounds of MAOPs. Finding the best type and intensity of light spectra is a crucial step for commercial-scale production of bioactive compound(s) of interest of MAOPs.

## Interaction of biotic and abiotic elicitors to produce bioactive compounds

Abiotic stresses, including water deficiency, high and low temperatures, salt, heavy metal accumulation and UV exposure, have significant effects on the quantity and quality of secondary metabolites accumulation in MAOPs (Khare et al. 2020; Mahajan et al. 2020). These stress-inducing factors have been refereed as "eustress factors" (Alvarado et al. 2019).

The simultaneous utilization of artificially induced abiotic stresses and exogenous application of defense-trigger biotic elicitors will be more effective than individually application of elicitors in increasing the in vitro accumulation of bioactive molecules in MAOPs. Biotic and abiotic elicitors can affect different metabolic pathways and molecules in target plants. Chitosan (50, 100 and 200 mg/L) along with MJ (100 µM) and methyl-b-cyclodextrin (0.7, 7 and 14 mM) elicitors were applied in hairy root cultures of *Scutellaria bornmuelleri*. Authors investigated individual and combined effects of applied elicitors and reported that MJ + chitosan (50 mg/L) led to the highest contents of chrysin (9.15-fold), wogonin (10.56-fold) and baicalein (13.25-fold) flavonoids (Gharari et al. 2020). In safflower (*Carthamus tinctorius* L.), chitosan (25 and 50 mg/L) and salicylic acid (50 and 100 mg/L) elicitors were applied on callus cultures under artificially induced salinity stress (NaCl, 1.5% w/v) and the highest content of total phenolics and total flavonoids were obtained by application of 50 mg/L salicylic acid and 25 mg/L chitosan. However, elicitation with SA was superior to chitosan (Golkar et al. 2019b). Razavizadeh et al. (2020) applied chitosan to reduce oxidative injury of salt stress, caused by different concentrations of NaCl, in callus and seedling cultures of ajowan (*Carum copticum* L.), and reported that applying 20 mg/L chitosan in MS medium containing 100 mM NaCl led to enhanced contents of thymol and *p*-cymene in both seedlings and calli cultures. Authors also reported that chitosan, under NaCl stress, reduced phenolic accumulation and antioxidant enzyme activities of cultures. These results indicated that chitosan, as compatible solutes, increased salt stress tolerance of ajowan in in vitro condition and subsequently led to increased content of thymol and *p*-cymene. Karamian et al. (2020) applied an abiotic stress-elicitor strategy in liquid in vitro culture of *Verbascum sinuatum*. For this purpose, drought stress was created artificially by adding PEG 6000 to liquid MS medium supplemented with different elicitors such as methyl jasmonate (MJA), salicylic acid (SA) and $TiO_2$ nanoparticles ($TiO_2$NPs). Authors reported that in severe drought stress (− 0.5 MPa), the highest total phenol and flavonoid contents were obtained through application of MJA (200 µM), whereas the highest anthocyanin and root saponin contents were obtained by adding $TiO_2$NPs (20 ppm) to the culture medium. Salinity stress (NaCl) along with sodium alginate (NaAlg) elicitation were applied to callus cultures of safflower and total flavonoids, total flavonols, catalase activity, phenylalanine ammonia-lyase, total antioxidant capacity and total phenolic content were increased significantly by application of 0.15% of NaAlg under salinity stress (Golkar et al. 2019a). The interaction effect of different elicitors, including NaCl (0 and 50 mM), salicylic acid (0 and 100 µM) and methyl jasmonate (0 and 100 µM), were assessed on solid and liquid callus cultures of stevia and the greatest contents of phenol and flavonoid were obtained with co-application of SA (100 µM) + NaCl (50 mM) in solid medium (Salmalian et al. 2019). In shoot culture of peppermint, volatile organic compounds from Rhizobacteria (*Bacillus amyloliquefaciens*) reduced the adverse effect of NaCl salt stress and increased the accumulation of phenols, antioxidants, MDA, menthone, menthol, and pulegone compounds under severe salinity stress (100 mM NaCl) (Del Rosario Cappellari et al. 2020). Nazir et al. (2020), investigated the combine effect of melatonin and UV-C on metabolite production and antioxidant potential in callus cultures of purple basil (*Ocimum basilicum* L. var *purpurascens*) and reported higher accumulation of caffeic acid (1.9-fold) in callus cultures treated with 4 mg/L melatonin + UV-C (20 min) than control. Recent reports of integrated biotic and abiotic elicitors to target secondary metabolites content of different MAOPs are presented in Table 2.

## Tolerance-enhancer additives along with defense-trigger biotic and abiotic elicitors

Biotic and abiotic stresses are well-known factors to changing accumulation and composition of secondary metabolites of plant materials. Despite their positive roles in increasing the secondary metabolites content, these stresses usually cause decreased biomass production, programmed cell death, oxidative stress, and production of reactive oxygen species ROS in plants. Tolerance to these inductive stresses can be increased through different mechanisms such as metabolic adjustments and applying some defense molecules (chemical enhancers) with stress tolerance-inducing properties. These tolerance-enhancer materials can reduce stress induced cell death by preventing accumulation of ROS (ROS

**Table 2** Examples of concomitant use of stress tolerance inducing agents and stresses inducing abiotic elicitors to impact secondary metabolites content of different medicinal, aromatic and ornamental plants

| Plant species | Culture system | Basal culture medium | Bioactive compound(s) of interest | Changed amount of targeted bioactive compound (±%) | Integrated stress response inducing and stress inducing elicitors to target metabolomic profile | Reference |
|---|---|---|---|---|---|---|
| Ajowan (*Carum copticum* L.) | Solid shoot and callus | MS | Thymol and *p*-cymene | – | CHT + NaCl | Razavizadeh et al. (2020) |
| | Solid shoot | MS | γ-terpinene, *p*-cymene, thymol | | SA + PEG, MJ + NaCl | Niazian et al. (2021) |
| Basil (*Ocimum basilicum* L.) | Solid callus | MS | Caffeic acid | + 1.9 | Melatonin + UV-C | Nazir et al. (2020) |
| *Fagonai indica* | Liquid suspension cell | MS | Phenols and flavonoids | – | 2,4-D, IBA, BA, TDZ, MJ, PAA + light regimes | Khan et al. (2018) |
| Peppermint (*Mentha piperita* L.) | Solid shoot | MS | Menthone, menthol, pulegone | – | mVOC + NaCl | Del Rosario Cappellari et al. (2020) |
| Safflower (*Carthamus tinctorius* L.) | Solid callus | MS | Phenols and flavonoids | – | CHT, SA + NaCl | Golkar et al. (2019b) |
| | Solid callus | MS | Phenols and flavonoids and antioxidant and phenylalanine ammonia-lyase | – | NaAlg + NaCl | Golkar et al. (2019a) |
| *Stevia rebaudiana* | Solid and liquid callus | MS | Phenol and flavonoid | – | SA + NaCl, MJ + NaCl | Salmalian et al. (2019) |
| *Verbascum sinuatum* | Liquid shoot | MS | Phenols and flavonoids and antioxidant and saponin | – | MJ, SA, $TiO_2$NPs + PEG | Karamian et al. (2020) |

*CHT* chitosan, *NPs* nanoparticles, *MJ* methyl jasmonate, *MS* Murashige and Skoog's medium, *mVOC* microbial volatile organic compounds, *PAA* phenyl acetic acid, *PEG* polyethylene glycol, *SA* salicylic acid

scavenging), preventing ethylene and gibberellin, activating antioxidant enzymes and detoxification capacity, and activating/regulating defense response signal pathways. PGRs, stress hormones, polyamines, compatible solutes, chitosan, chlormequat chloride (CCC), non-enzymatic cellular antioxidants, silver nitrate, colchicine, ethylene inhibitors, gibberellin inhibitors (such as triiodobenzoic acid, benzothiazole-2-oxyacetate, and N-dimethylamino succinamic acid), active charcoal, polyvinylpyrrolidone (PVP), boron ($H_3BO_3$), calcium ($Ca^{2+}$), nitric oxide, and cell wall remodeling agents (such as arabinogalactan proteins) are other examples of these chemical enhancers. Niazian and Shariatpanahi, (2020) have described the mechanisms by which these chemical additives increase stress tolerance of different plant species. These chemical additives can be used in combination with biotic and abiotic elicitors and physical stresses in different types of in vitro and in vivo cultures to enhance accumulation of valuable bioactive compounds.

Regulating the primary metabolites and accumulating soluble osmotic adjustment substances for cell membrane integrity, is one of the plants strategies to survive during stress conditions. Under stress, plant cells accumulated large amounts of low molecular and highly soluble organic compounds including sugar, amino acids, nitrogen and sulphur compounds. These compatible solutes act as osmoprotectant components and modulate wide range of biological processes by interacting with other important elements of the cells such as antioxidative enzymes, stabilized proteins and plasma membranes (Niazian and Shariatpanahi 2020). Proline, glycine betain, polyols and trehalose are some of these ROS scavenging compatible solutes that can enhance tolerance to abiotic stresses. In *Brassica napus* (L.), enhanced efficiency of microspore embryogenesis under cold stress stress using proline additive has been reported (Ahmadi and Shariatpanahi 2015). Results indicate to it that proline and other compatible solutes can also be used in stress-induced studies for production of secondary metabolites. Yu et al. (2019), applied SA, NaCl and $AgNO_3$ elicitors in cell suspension culture of *Salvia miltiorrhiza* Bunge and showed that sucrose and proline were significantly increased in cells

treated with $AgNO_3$. Different concentrations of proline (1, 5, 25, 50, 100 and 200 mg/L) were applied as biotic elicitor in in vitro callus cultures of chitrak and a 1.41-fold increase in plumbagin accumulation was observed using 50 mg/L proline (Singh et al. 2020b).

One of the ethylene inhibitor materials that widely applied as elicitor is $AgNO_3$ (Mahendran et al. 2018). Another tolerance enhancer additive that has also been applied as elicitor is melatonin (N-acetyl-5-methoxytryptamine). Melatonin is a well-known animal hormone that also has been reported in plants and its role in enhance plants tolerance against biotic and abiotic stresses has been documented (Zhang et al. 2015; Nawaz et al. 2016). Significant effects of abiotic stresses, such as drought, salt, and cold, on the expression patterns of melatonin biosynthetic genes has been reported (Ahn et al. 2021). Foliar application of melatonin improved glutathione content, redox state and increased essential oil production of two *Salvia* species (*Salvia nemorosa* L., and *Salvia reuterana* Bois) under drought stress condition (Bidabadi et al. 2020). Melatonin on pepper (*Capsicum annum* L.) plants enhanced the activities of antioxidant enzymes and expression of chitinase gene (*CaChiIII2*) under the infection of *Colletotrichum gloeosporioides* disease (Ali et al. 2020). The positive effect of melatonin elicitation on rosmarinic acid accumulation has been reported in callus culture of rosemary (*Rosmarinus officinalis* L.) (Coskun et al. 2019) and sweet basil (*Ocimum basilicum* L.) (Duran et al. 2019).

## Machine learning algorithms for modeling and optimizing in vitro production of valuable bioactive compounds

Different plant species and genotypes reported various responses to the alteration of in vitro conditions when valuable bioactive compounds are considered. Genotype-dependency leads to different results to applied elicitors among various plant genotypes. Yeow et al. (2020) compared the effects of different light intensity (cool white light, far red, green, blue, red and blue-red LED) with amino acids (L-leucine, glycine and proline) on secondary metabolites production in protocorm-like-body (PLB) cultures of a *Dendrobium* hybrid orchid (*Dendrobium Enopi* × *Dendrobium* Pink Lady). They reported significant effect of green and blue-red LEDs on accumulation of phenolics and flavonoid secondary metabolites, while applied amino acids had no significant stimulating effect on secondary metabolites. These results revealed the differential effect of two types of elicitors on secondary metabolites accumulation of a specific plant genome. This does not mean that elicitors are plant-specific; however, the different types of elicitors may lead to different chemical profile of a certain plant genome. Therefore, we need to find elicitors that increase the accumulation of useful chemical component(s). Finding the optimum type, concentration and combination of elicitors is a complex task. In addition to mentioned precursors, elicitors and tolerance enhancer additives, there are other culture media parameters that can affect the accumulation of bioactive compounds. Jeet et al. (2020) investigated the effect of culture media (B5, MS, WH, NN and SH), PGRs (2,4-D and FAP) (in 0.1, 0.3, 0.4, 0.5, 0.6, 0.7 and 1.0 mg/L concentrations), carbon sources (sucrose, glucose, galactose and fructose), sucrose concentrations (1%, 2%, 3%, 4% and 5% w/v) and pH of the medium (4.8, 5.0, 5.8, 6.0 and 6.8) on the accumulation of indole alkaloids in the callus culture of *Alstonia scholaris*. They reported that MS medium supplemented with 0.3 mg/L of 2,4-D plus 0.5 mg/L of FAP and 3% (w/v) sucrose with medium pH 5.8 was the optimum condition for the accumulation of echitamine, acetylechitamine, tubotaiwine and picrinine indole alkaloids. These results indicate other parameters (culture media optimization) than culture types, elicitors and precursors can affect the results of culture performance and productivity of valuable plant bioactive compounds.

The interaction of culture systems (liquid and solid differentiated and undifferentiated cultures), elicitors and precursors with in vitro culture medium parameters—basal culture medium, PGRs, carbon sources, additives and pH—creates a multi-factorial condition with a non-linear relationships between all the different parameters (Fig. 1). Classical statistical methods, such as factorial experiments, are not capable to discern a high volume of non-linear and non-deterministic data. In such situations, researchers benefit from machine learning methods to interpret their findings more accurately (Niazian and Niedbała 2020). Different shallow and deep non-linear machine learning algorithms, including random forest (RF), support vector machines (SVMs) and neural networks, have been applied by researcher to interpret different multi-factorial in vitro experiments (Niazian et al. 2019b; Hesami and Jones 2020; Hesami et al. 2020a). Among different computational approaches, response surface methodology (RSM) and artificial neural network (ANN) have great potential for optimizing in vitro culture performance and improving the production of various bioactive compounds (Khattab and Farag 2020). Kaur et al. (2020) applied RSM and ANN for modeling and optimizing salicylic acid and chitosan elicitation to produce bitter secoiridoid and xanthone glycosides in shoot cultures of *Swertia paniculata*. Authors reported that, according to optimization results, 0.170–0.435% of amarogentin, 1.020–4.987% of swertiamarin and up to 2.550–4.357% of mangiferin can be obtained with varied range of SA (1–20 mM) and chitosan (1–20 mg/L). In addition, ANN modeling was more accurate than RSM in optimization of elicitors to promote secoiridoid and xanthone glycoside production, based on root mean square error (RMSE), absolute average deviation (AAD) and regression coefficient ($r^2$) statistics (Kaur et al. 2020). In another study, Salehi et al. (2020a) applied

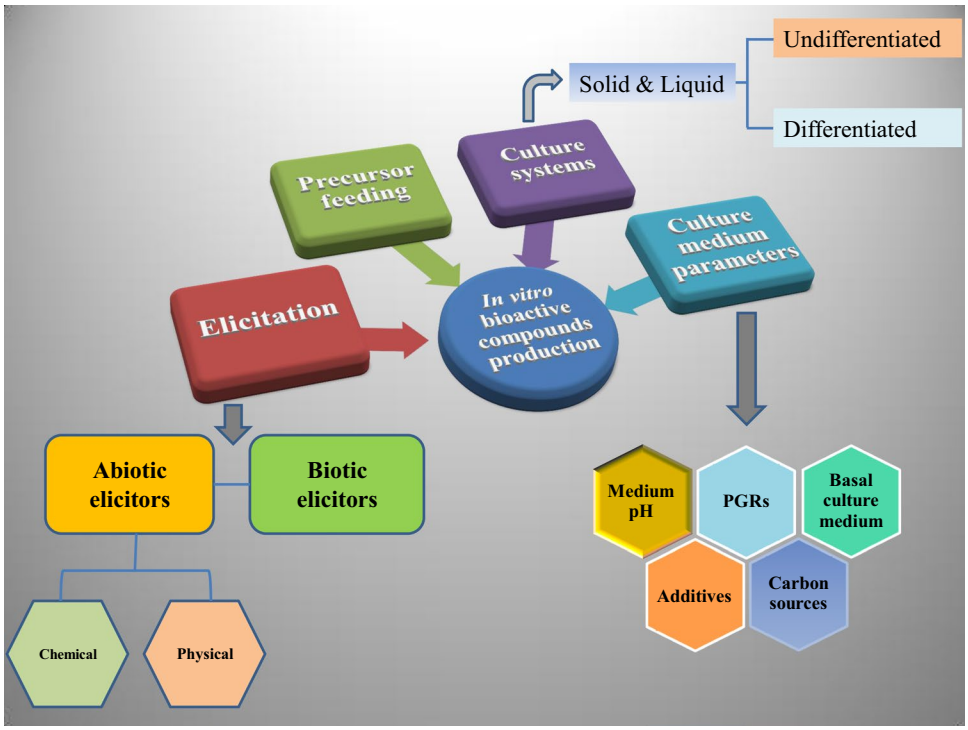


**Fig. 1** Factors affecting in vitro production of bioactive compounds of medicinal, aromatic and ornamental plants

multilayer perceptron-genetic algorithm (MLP-GA) for selecting the optimal conditions for maximum paclitaxel biosynthesis in cell suspension culture of *Corylus avellana* under the effect of fungal elicitors (cell extract and culture filtrate derived from fungal strain HEF17 isolated from *C. avellana*). For this purpose, cell extract and culture filtrate concentration levels, elicitor adding day, and cell suspension culture harvesting time were considered as inputs of the model and dry weight, intracellular, extracellular and total yield of paclitaxel and extracellular paclitaxel portion were assigned as outputs of the model. Authors reported that developed MLP-GA was more accurate than classical regression models and can be used as promising tool for predicting fungal elicitor-assisted paclitaxel biosynthesis in cell suspension cultures of *C. avellana* (Salehi et al. 2020a). These results indicate the power of machine learning algorithms in modeling and optimizing elicitation experiments. Using these powerful computational methods, researchers are able to manage wide range of factors, including culture media optimization, precursors, biotic and biotic elicitors, physical stresses and tolerance enhancer additives, and their interactions in different culture systems for large-scale in vitro production of valuable bioactive compounds of MAOPs (Fig. 2).

## Conclusion

In vitro culture system, as one of the simplest plant biotechnology-based methods, has great potential for safe and sustainable production of valuable bioactive compounds in MAOPs. Different in vitro culture systems, including undifferentiated and differentiated solid and liquid cultures, are strategic approaches for secondary metabolites production of MAOPs. These culture systems can lead to different results in terms of bioactive compounds synthesis and production. Species-dependent yield and difficulty in scaling-up production are the current limitations and problems of in vitro strategies for sustainable production of bioactive compounds of MAOPs. Precursor feeding, elicitation and culture media optimization (basal culture medium, PGRs, carbon sources, additives and pH) are the applicable strategies to solve the mentioned problems in in vitro production of pharmaceutical secondary metabolites of MAOPs. There are many reports about the positive effect of precursor feeding and elicitation on increasing the content of secondary metabolites of MAOPs in different in vitro culture systems.

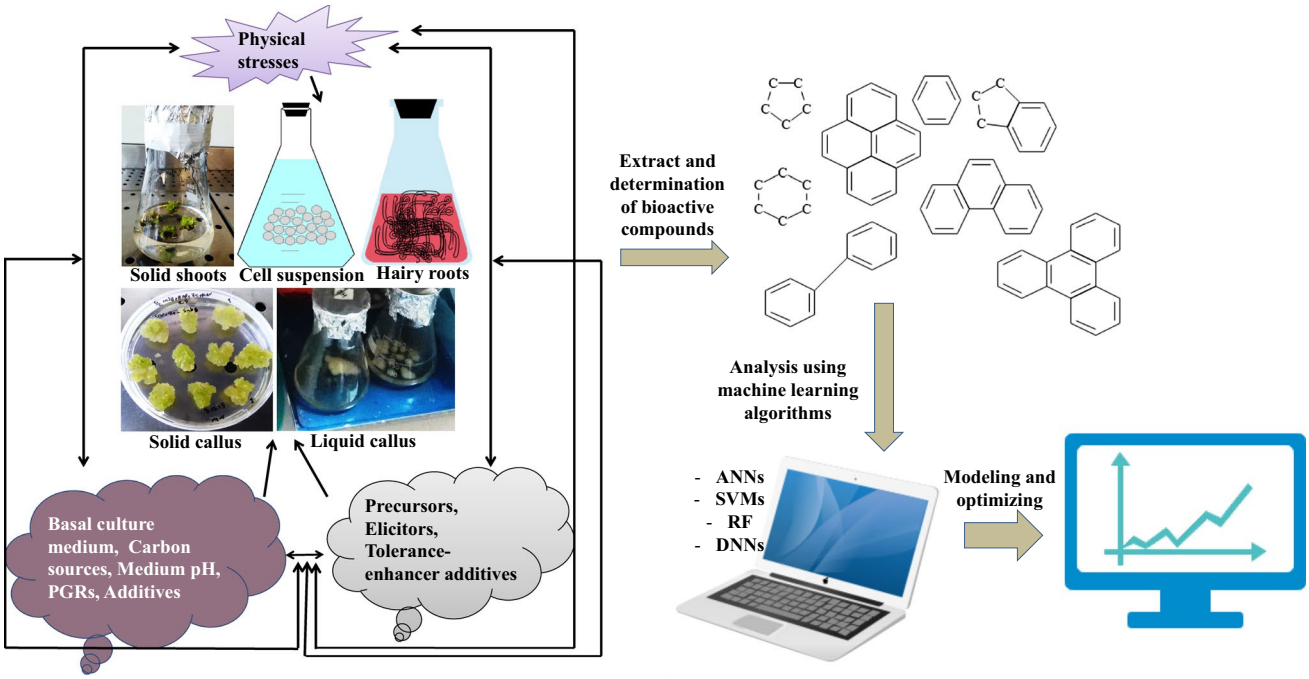


**Fig. 2** Modeling and optimizing in vitro production of bioactive compounds of medicinal, aromatic and ornamental plants using machine learning algorithms

The important role of secondary metabolites as defense elements of plants and their involvement in protective functions against biotic and abiotic stress conditions is well documented. Therefore, stimulating and activating the defense mechanisms is an effective strategy to increase in vitro production of these defense elements. This can be achieved with the use of biotic and abiotic elicitors. These biotic and abiotic elicitors can be divided into stress-inducing and stress-tolerance-inducing materials. Compounds like NaCl and PEG are stress-inducers; however, biotic elicitors like SA and chitosan are stress-tolerance inducers, which alleviate the negative effects of abiotic stress through the modulation of primary metabolites and osmoprotectants like proline. The integrative application of stress inducing and stress tolerance-inducing elicitors may lead to more favorable results than just their individually application.

Machine learning algorithms, as cutting-edge techniques to detect critical steps and conditions in specific procedures, are able to manage and process a large volume of data generated taking into account multiple factors (e.g. culture systems, precursor feeding, elicitation and culture medium parameters). Therefore, artificial intelligence systems could help researchers to reach conclusions from empirical experiments and manage the interaction of different tools available to increase in vitro production of secondary metabolites of MAOPs.

***Author contribution statement*** MN conceptualized this study, wrote the original draft, reviewed, and edited it. PS reviewed, edited and revised the manuscript for important intellectual content.

**Data availability** Data sharing not applicable to this article as no datasets were generated or analyzed during the current review study.

## Declarations

**Conflict of interest** The authors declare that there are no conflicts of interests. All the authors read and approved the manuscript in its final form.

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## Authors and Affiliations

**Mohsen Niazian**[1] · **Paolo Sabbatini**[2]

[1] Field and Horticultural Crops Research Department, Kurdistan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Jam-e Jam Cross Way, P. O. Box 741, Sanandaj, Iran

[2] Department of Horticulture, Michigan State University, Plant and Soil Sciences Building, East Lansing, MI 48824, USA