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Nanoelicitation of specialized plant secondary metabolites by plant tissue culture: A review

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ABSTRACT

Plant secondary metabolites (SMs) are high-value, commercially significant chemical compounds produced by plants in response to various biotic and abiotic stimuli which are having high bioactive potential and are utilized in pharmaceutical, nutraceutical, cosmeceutical industries and more. But due to certain environmental, economic and geographic reasons, extracting such high-value metabolites in large-scale from intact plants are not practical. Due to its high demand and less availability, biotechnological approaches such as plant tissue culture have gained significant interest for increased production of commercially important SMs while preventing overexploitation and extinction of the source plants. To improve the productivity, certain methods like elicitation is carried out in cultures using various biotic and abiotic elicitors. Among them, nanoparticles (NPs) shows promising results in eliciting SMs in tissue culture systems due to their customizable physicochemical properties. Biogenic NPs are eco-friendly, non-toxic, economic and efficient than chemically and physically synthesized ones. Therefore, at present, there is an increased trend in utilizing such green synthesized NPs as elicitors. Such NPs with smaller size can easily penetrate the cells and cause significant biochemical and molecular changes within the plant cell leading to the activation of secondary metabolic pathways and thus resulting in enhanced production of SMs.

1. INTRODUCTION

Secondary metabolites (SMs), in particular, which are remarkable sources of value-added bioactive molecules, provide the basis for the pharmacological capabilities of plants, which have been employed as medicines throughout history [1]. SMs are a huge and diverse class of compounds, some of which are probably unimportant byproducts of metabolic pathways brought on by different enzyme activity [2]. SMs are formed in response to many types of stress it faces and have a complicated chemical makeup that helps to carry out diverse physiological activities in plants [1]. About 200,000 known SMs are found in plant species and are crucial for survival, reproduction, forming symbiotic associations, climatic tolerance, and disease resistance [3,4]. Plant secondary metabolism is typically activated and reconfigured during acclimatization and adaptation to adverse environmental conditions, and stimulation of this specialized metabolism to combat these environmental stresses results in the production of various classes of plant SMs, including phenolic compounds (coumarin, lignin, furanocoumarins, flavonoids, isoflavonoids, and tannins), terpenes (monoterpenes, sesquiterpenes, diterpenes, triterpenes, and polyterpenes), nitrogen-containing SMs (alkaloids, cyanogenic glycosides, glucosinolates, and non-protein amino acids), and sulfur-containing SMs (glucosinolates, phytoalexins) [5,6]. They are having wide applications in human use and are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives, and have a variety of biological activities. Therefore, the majority of SMs, including terpenes, phenolics, and alkaloids, are highly sought after in the biopharmaceutical, cosmeceutical, and nutraceutical industries [7,8].

Few significant plant-based compounds with uncomplicated chemical structures can be produced using the chemosynthesis method, but many, like alkaloids, are challenging to synthesize by the chemosynthesis method, or their production costs outweigh their commercial availability [9]. From intact plants grown in nature, many of these specialized compounds can be obtained. The plant raw materials from which several of these highly valuable substances are derived are getting scarcer due to ecological, political, or geographic factors. A metabolite's production tends to be confined to a single species or genus and may only be initiated during a specific growth or developmental stage of a plant, or may be related to the season, stress, or nutrient availability [9,10]. Different types of stresses, including biotic and abiotic stresses that a plant has to encounter, make it difficult to ensure and maintain a steady supply of desirable plant SMs, and also, their subsequent processing is challenging. These drawbacks make it difficult to isolate SMs from intact plants [11]. The restricted availability of the compounds is one of the key issues with the commercial supply of molecules derived from plants. Most of the time, SMs make up less than 1% of the dry weight (DW) of plant cells,

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and occasionally even less, as is the case with the paclitaxel content of *Taxus* bark, which is only 0.01% [12].

The elevated demand in the marketplace and limited yield of pharmaceutically significant phytochemicals have sparked interest in biotechnological methods to increase the yield of SMs [13]. For SM production under controlled conditions, plant cell culture systems (cell suspension, hairy/adventitious root (AR) cultures, and callus cultures) have emerged as an appealing substitute [11]. Utilizing a plant cell culture technology may be able to lower the overexploitation of therapeutic plants that are in danger of extinction, and also those that are very rare [14]. By using plant tissue culture (PTC) techniques, cultures may be generated anywhere in the world, regardless of the conditions needed for plant growth. They are also free of pathogens and insects, preventing the need for pesticides and herbicides [9]. Recently, various techniques have been developed to increase the production of important plant SMs employing plant *in vitro* cultures, including cell line selection, precursor feeding, cell immobilization, biotransformation, metabolic engineering, synthetic biology, and elicitation [15].

Through elicitation employing a variety of biotic and abiotic substances, a simpler method to improve SM generation in plant cell culture systems can be achieved [16]. These elicitation strategies range from media manipulations through environmental stress and chemical elicitors, and it shows encouraging results in terms of boosting the yield of SMs in cell cultures of different medicinal plants [17]. Use of nanoparticles (NPs) as elicitors is giving assurance as more potent substitutes to other elicitors due to their customizable physicochemical properties and the physiological, molecular, biochemical, and metabolic responses they generate [18,19]. There are many previous studies reporting the use of NPs in order to positively impact the yield of useful SMs in plants. NPs consisting of metals, metal oxides, non-metallic, magnetic, polymeric, bi-metallic, carbon-based, silica, biopolymer, core-shell structures, and nanocomposites are used for the elicitation of SMs in plant cell culture systems [11]. Because of their smaller size, ease of penetration, and consequent ease in manipulating the cell's machinery to generate increased phytochemicals, nanomaterials have been used and studied widely over the past few years. PTC has utilized nanomaterials for a variety of goals, including growth, organogenesis, transformation, and the induction of SMs in many plants [13].

2. SECONDARY METABOLITE PRODUCTION BY PLANT TISSUE CULTURE TECHNIQUES

The demand for natural and organic products has grown as consumers become more aware of lifestyle choices. Due to its health advantages over synthetic medications, as well as in terms of cost and safety, fascination with medicinal plants has grown over the past two decades [1]. Due to limitations in topography and environment, isolation of plant SMs on a large scale from naturally existing intact plants is restricted [15]. Traditional SM isolation techniques take a long time since it takes plants years to develop to a stage where they can produce the desired metabolites [20]. It also has a number of drawbacks, including low yields and concentration changes brought on by regional, seasonal, and environmental variations [7].

Utilizing PTC procedures for the quick and effective production of SMs for commercial use is an alternative strategy to overcome such a dilemma. A need to concentrate research on raising output by applying PTC approaches and improving their large-scale production using bioreactors has resulted from the extensive usage of SMs in numerous industrial sectors [1]. *In vitro* propagation of plants or the *in vitro* culture of plant organs (usually roots) or callus are reported to be highly capable of providing plant materials that produce SMs [21]. It facilitates the bulk propagation of plants in controlled environmental conditions without regard to the seasons [22]. In addition, it facilitates the year-round production of huge numbers of homogeneous plants, disease-free propagules, and a significant increase in multiplication rates over traditional horticultural propagation approaches [21].

The process for producing *in vitro* SMs involves two steps: aggregation of biomass and the synthesis of SMs [1]. The following are procedures for producing bioactive SMs using plant *in vitro* culture: A) surface sterilization of the plant material, followed by establishing *in vitro* cultures. Organ culture material or callus can also be used. *Agrobacterium rhizogenes* is used to produce hairy roots by infecting sterilized donor plants or *in vitro* cultured materials. B) Multiplication of obtained primary callus/organs/roots, their initial selection, followed by establishing liquid cultures. C) High-yielding lines are chosen, and culture conditions are optimized (nutrient medium constituents, inoculum concentration, temperature, light, agitation, and aeration). To increase productivity even further, techniques like elicitation, precursor feeding, or immobilization are used. D) Bioreactor types depending on the culture: stirred tanks, airlift, and bubble column reactors for cell suspensions; mist or spray reactors and temporary immersion systems for organ cultures, including hairy roots, are used for further scale-up of SM production [23].

The entire procedure must be carried out in an aseptic environment utilizing a laminar flow cabinet equipped with a high-efficiency particulate air (HEPA) filter in order to accomplish a successful culture. The environment has extensively contaminated the source of the explants; thus, the portion that is to be removed for use is disinfected in a disinfectant, often alcohol, sodium, or calcium hypochlorite or mercuric chloride [24]. The cultures can be initiated from either seeds that have been germinated aseptically or from pieces of the entire plant. The explants are then inoculated on a nutrient-rich semi-solid medium that contains plant growth regulators and are grown in a controlled environment [23]. Two key characteristics of plant cells—cell plasticity and totipotency—support the growth of explants. These two characteristics of plant cells probe the ability of living cells to differentiate into new, genetically similar cells that can then become tissues, organs, and whole individuals [25].

Cell cultures (cell suspensions, protoplasts, or gametic cells), tissue cultures (callus or differentiated tissues), or organ cultures (shoots, roots, or zygotic embryos) can be created based on the starting material and the nutrient medium [26]. For the development of SMs for commercial use, callus, cell suspension, shoot, and root cultures have been used most frequently [1]. The sequential steps involved in SM production by PTC techniques are schematically represented in Figure 1. Several research reports have showcased successful outcomes regarding the production of important SMs using PTC techniques, as listed in Table 1. A comparative analysis for the enhancement of bioactive compounds in *Ocimum basilicum, Ocimum sanctum,* and *Ocimum gratissimum* by cell culture was carried out, and found that *O. basilicum* produced 33 mg GAE g^{-1} DW, 220 mg g^{-1} DW, and 450 mg g^{-1} DW of total phenolic, alkaloid, and terpenoid content, respectively. Likewise, *O. sanctum* synthesized 33.3 mg GAE g^{-1} DW, 330 mg g^{-1} DW, 430 mg g^{-1} DW, and *O. gratissimum* synthesized 44.0 mg GAE g^{-1} DW, 450 mg g^{-1} DW, 420 mg g^{-1} DW of total phenolic, alkaloid, and terpenoid content, respectively [27]. Cell suspension culture of *Tinospora cordifolia* showed a 5.57-fold (3.077 mg g^{-1} DW) increase in berberine production, while cell suspension culture of *Vernonia anthelmintica* showed a 9.4-fold (1.104 mg g^{-1} DW) increase in rhamnetin content [28,29]. Kuo *et al*. [30] studied the effect of *in vitro* cultured plants on cucurbitacin I and cucurbitacin

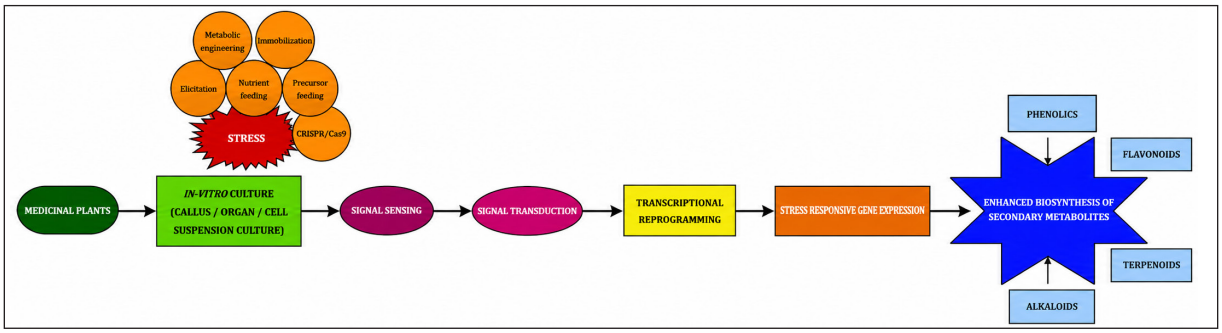

Figure 1. Secondary metabolite production by plant tissue culture techniques.

Table 1. Some bioactive SMs obtained via PTC systems.

Source plant species	SM	Product yield	Type of culture	Reference
Ajuga bracteosa	TPC	4.5 mg GAE g^{-1} DW	Shoot culture	[31]
	TFC	3.5 mg QE g^{-1} DW		
Aquilaria agallocha	Cucurbitacin I	0.356 mgg^{-1}	*In-vitro* plantlets	[30]
	Cucurbitacin E	0.972 mgg^{-1}		
Argemone mexicana	Berberine	1.4 mg g^{-1} DW	Callus culture	[32]
Artemisia annua	TPC	147.98 mg GAE g^{-1} DW	Callus culture	[33]
Bacopa floribunda	Bacoside A3	1.01 mg g^{-1} DW	*In-vitro* plantlets	[34]
	Bacopaside X	1.23 mg g^{-1} DW		
	Bacopaside II	43.62 mg g^{-1} DW		
	Bacosaponin C	0.19 mg g^{-1} DW		
	Stigmasterol	7.69 mg g^{-1} DW		
Bletilla striata	p-hydroxybenzyl alcohol	1.05 mg g^{-1} DW	Callus suspension culture	[35]
	Dactylorhin A	19.0 mg g^{-1} DW		
	Militarine	6.25 mg g^{-1} DW		
	Coelonin	0.3323 mg g^{-1} DW		
Boerhavia diffusa	Boeravinone-B	0.673 mgg^{-1} DW	Callus culture	[36]
	TPC	63.48 mg GAE g^{-1} DW		
	TFC	30.22 mg QE g^{-1} DW		
Boesenbergia rotunda	Pinostrobin	17.97 mg g^{-1} DW	Shoot culture	[37]
	TPC	86.01 mg GAE g^{-1} DW		
	TFC	166.6 mg QE g^{-1} DW		
Capsicum chinense	Capsaicin	2.87 mg g^{-1} FW	Cell suspension culture	[38]
	Dihydrocapsaicin	1.03 mg g^{-1} FW		
Caralluma tuberculata	TPC	3.0 mgg^{-1} DW	Callus culture	[39]
	TFC	1.8 mg g^{-1} DW		
Catharanthus roseus	Total alkaloid content	5.67 mg g^{-1} DW	Cell suspension culture	[40]
Centella asiatica	Centellosides	0.134 mg g^{-1} DW	Cell culture	[41]
Digitalis purpurea	TPC	5.43 mg g^{-1} DW	Callus culture	[42]
	TFC	3.27 mg g^{-1} DW		
Eclipta alba	TPC	57.8 mg g^{-1}	Callus culture	[43]
	TFC	11.1 mg g^{-1}		
Ficus carica	TPC	55.95 mg GAE g^{-1}DW	Hairy root culture	[44]
	TFC	3.9355 mg QE g^{-1} DW		

Continued

Source plant species	SM	Product yield	Type of culture	Reference
Glycine max	Isoflavones	21.027 mg g^{-1} DW	Cell suspension culture	[45]
	TPC	0.1108 mg g^{-1} FW		
Hyoscyamus niger	Scopolamine	0.64 mg g^{-1}	AR culture	[46]
	Hyoscyamine	0.46 mg g^{-1}		
	TPC	19.33 mg g^{-1}		
Narcissus tazetta	Galantamine	0.158 mg g^{-1} DW	Shoot culture	[47]
	TPC	1.070 mgg^{-1} DW		
Ocimum basilicum	TPC	33 mg GAE g^{-1} DW	Cell culture	[27]
	Total alkaloid content	220 mg g^{-1} DW		
	Total terpenoid content	450 mg g^{-1} DW		
Ocimum basilicum	Rosmarinic acid	0.062 mgg^{-1} DW	Hairy root culture	[48]
	TPC	0.0623 mgg^{-1} DW		
Ocimum gratissimum	TPC	44.0 mg GAE g^{-1} DW	Cell culture	[27]
	Total alkaloid content	450 mg g^{-1} DW		
	Total terpenoid content	420 mg g^{-1} DW		
Ocimum sanctum	TPC	33.3 mg GAE g^{-1} DW	Cell culture	[27]
	Total alkaloid content	330 mg g^{-1} DW		
	Total terpenoid content	430 mg g^{-1} DW		
Oplopanax elatus	Flavonoids, Phenolics	53.87 mg g^{-1} DW	AR culture	[49]
		30.10 mg g^{-1} DW		
Paris polyphylla	TPC	49.66 GAEg^{-1} DW	Callus suspension culture	[50]
	TFC	34.67 QE g^{-1} DW		
	TTC	57.66 TAE g^{-1} DW		
Prunella vulgaris	Polyphenolics	7.62 mg g^{-1} DW	Cell suspension culture	[51]
Silybum marianum	t-resveratrol	12 mg l^{-1}	Cell suspension culture	[52]
Taxus media	Taxane	103.5 mg l^{-1}	Cell culture	[53]
Tinospora cordifolia	Berberine	5.05 mg g^{-1}	*In-vitro* plantlets	[54]
	Palmatine	3.00 mg g^{-1}		
Tinospora cordifolia	Berberine	3.077 mgg^{-1} DW	Cell suspension culture	[28]
Urginea maritima	TPC	1.02 mg GAE g^{-1} DW	Callus culture	[55]
Vernonia anthelmintica	Rhamnetin	1.104 mg g^{-1} DW	Cell suspension culture	[29]
Vitis vinifera	Resveratrol	1.33 mg g^{-1} DW	Cell suspension culture	[56]
	ε-viniferin	6.2 mg g^{-1} DW		

DW: Dry weight; FW: Fresh weight; TPC: Total phenolic content; TFC: Total flavonoid content; TTC: Total tannin content; GAE: Gallic acid equivalents; QE: Quercetin equivalents; TAE: Tannic acid equivalents. All values were reported as per original studies. Wherever possible, units were standardized to mg. g^{-1} DW, Values originally reported in µg. g^{-1} and g. g^{-1} were converted accordingly. FW were not converted to DW due lack of moisture content data. Values in mg. l^{-1}represent concentration in culture medium and are not directly comparable with biomass-based measurements. Where the basis (DW/FW) was not specified in the original studies, values are presented as reported without assumption.

E production in *Aquilaria agallocha* and found that it enhanced production and observed 0.356 and 0.972 mg g^{-1} yield, respectively. Otari *et al*. [34] studied *in vitro* plantlets of *Bacopa floribunda* and reported an increase of Bacoside A3 (1.01 mg g^{-1} DW), Bacopaside X (1.23 mg g^{-1} DW), Bacopaside II (43.62 mg g^{-1} DW), Bacosaponin C (0.19 mg g^{-1} DW), and Stigmasterol (7.69 mg g^{-1} DW). Enhancement of total phenolic content (TPC) and total flavonoid content (TFC) was studied by various scientists and reported 4.5-mg GAE g^{-1} DW and 3.5-mg QE g^{-1} DW of TPC and TFC, respectively, in shoot culture of *Ajuga bracteosa*; 55.954-mg GAE g^{-1} DW, 3.9355-mg QE g^{-1} DW, respectively, in hairy root culture of *Ficus carica*; 5.43-mg g^{-1} DW, 3.27-mg g^{-1} DW, respectively, in callus culture of *Digitalis purpurea*; 3.0-mg g^{-1} DW, 1.8-mg g^{-1} DW, respectively, in callus culture of *Caralluma tuberculata* and 57.8-mg g^{-1} DW, 11.1-mg g^{-1} DW, respectively, in callus culture of *Eclipta alba* [31,44,42,39,43]. Berberine (5.05 mg g^{-1}) and palmatine (3.00 mg g^{-1}) were obtained from *in vitro T. cordifolia* plantlets [54]. AR culture of *Hyoscyamus niger* exhibited increased production of Scopolamine (0.64 mg g^{-1}), Hyoscyamine (0.46 mg g^{-1}), and TPC (19.33 mg g^{-1}) [46]. Escrich *et al*. [53] observed a 15-fold increase in taxane production by cell culture of

Taxus media. Improved production of centellosides by cell culture of *Centella asiatica*, berberine by callus culture of *Argemone mexicana,* and t-resveratrol by cell suspension culture of *Silybum marianum* were reported [41,52,32]. Increased total phenolic content (147.98, 1.02 mg GAE g^{-1} DW) was obtained by callus culture of *Artemisia annua* and *Urginea maritima,* respectively [33,55]. 2.44, 1.81, and 1.25 times greater amounts of pinostrobin, total phenolics, and flavonoids were reported by Jirakiattikul *et al*. [37] from shoot culture of *Boesenbergia rotunda*. Alkaloid content (5.67 mg g^{-1} DW) was found to be enhanced by cell suspension culture of *Catharanthus roseus* [40]. 1.05 mg g^{-1} DW of p-hydroxybenzyl alcohol, 19.0 mg g^{-1} DW of Dactylorhin A, 6.25 mg g^{-1} DW of Militarine, and 0.3323 mg g^{-1} DW of Coelonin were obtained by *Bletilla striata* callus suspension culture [35]. Park *et al*. [47] synthesized 0.158 mg g^{-1} DW of galantamine and 1.070 mg g^{-1} DW of TPC by shoot culture of *Narcissus tazetta*. Rawat *et al*. [50] reported increased production of TPC (49.66 GAE g^{-1} DW), TFC (34.67 QE g^{-1} DW), and total tannin content (TTC) (57.66 TAE g^{-1} DW) in callus suspension of *Paris polyphylla*. Total phenolics and rosmarinic acid contents were enhanced by hairy root culture of *O. basilicum* [48]. Production of boeravinone-B, total phenolics, and flavonoids was reported to be enhanced by callus culture of *Boerhavia diffusa* [36]. Resveratrol and ε-viniferin yield were increased by 26 and 620-fold in cell suspension culture of *Vitis vinifera* by Wang *et al*. [56]. *Capsicum chinense* cell suspension culture improved the quantity of capsaicin (2.87 mg g^{-1} FW) and dihydrocapsaicin (1.03 mg g^{-1} FW) [38]. A 10-fold increase in isoflavones was reported in studies of Jeong *et al*. [45] by cell suspension culture of *Glycine max*. They also reported an enhanced production of total phenolics (0.1108 mg g^{-1} FW). Fazal *et al*. [51] reported that cell suspension of *Prunella vulgaris* produced 7.62 mg g^{-1} DW of polyphenolics [51]. 1.66 times higher flavonoid content and 1.49 times higher phenolics were obtained by AR culture of *Oplopanax elatus* [49].

3. STRATEGIES TO INCREASE SECONDARY METABOLITES YIELD

Despite being widely established, PTC approaches for the generation of SMs are still only applied to a few processes in large-scale manufacturing. Key strategies (Fig. 2) used to boost production of SM in plant cell cultures are: selection of high-yielding cell lines through screening and optimization to identify the most productive strains; optimization of culture conditions, including medium composition, pH, temperature, light, and oxygen levels to create an ideal environment for metabolite production; addition of elicitors, such as fungal extracts, signaling molecules, and abiotic stressors, to trigger defense responses and metabolite production; feeding of precursors and intermediates to provide building blocks and boost biosynthetic pathways; employing permeabilization techniques, such as ultrasonication, to increase cell permeability and metabolite release; use of two-phase culture systems to continuously remove and sequester products avoiding feedback inhibition; addition of adsorbents like activated charcoal or resins to capture and remove metabolites from the medium; application of metabolic engineering to enhance biosynthetic pathways or block competing pathways; *in situ* removal of the products from culture to increase the accumulation of SMs which may be limited by feedback inhibition; immobilization technique by using a simple gel matrix which have reported to increase metabolite production, as well as ease the process of extraction and purification of the resulted products; and scale-up using bioreactors with optimized designs for plant cell culture at a larger scale. Another method that can be used for enhancing SM production is genome editing using CRISPR/Cas9 technology. These strategies aim to overcome the challenges of low productivity and enable commercial-scale production of valuable plant-derived pharmaceuticals using cell suspension cultures [15,20,57,58].

4. ELICITATION

Plant cells generate SMs as a defense mechanism against infectious pathogenic elicitors, as well as in response to environmental stresses during *in vivo* growth. As a result, there is growing interest in using substances to help develop plant cells, tissues, or organs to mount a defense response in order to increase the production of bioactive chemicals under *in vitro* cultures. This process is referred to as elicitation [57]. The technique of SM elicitation involves the exogenous administration of elicitors in cell and tissue cultures. Changes to the culture media, such as the exogenous administration of elicitors or precursors or modifying external factors, can result in the induction of the stress response and an increase in the synthesis of essential metabolites [18].

Chemical, physical, or biological factors that can cause physiological as well as biochemical alterations by inducing stress in the target living things are known as elicitors [59]. On application of such elicitors into the target system, it triggers and enhances the buildup of the specified metabolites even when it is applied in a very small amount [60]. They have been used exogenously and have been demonstrated to stimulate secondary metabolism and encourage the accumulation of desirable metabolites in a variety of plant species. The biosynthetic pathways can be affected by a variety of elicitors in different ways [20].

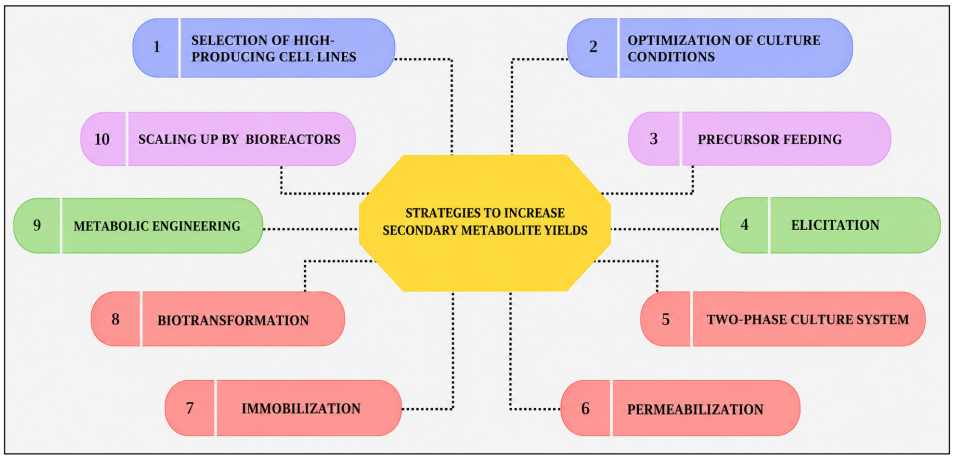

Figure 2. Strategies to increase SM yields.

Elicitors can be classified as biotic or abiotic, as well as general and race-specific elicitors [10]. Abiotic elicitors are components of non-biological sources, including the majority of inorganic chemicals, such as salts of heavy metals, metal ions, and metal oxides, as well as physical stresses like cold shock, UV, osmotic, water stress, light, temperature shift, and others [61,62]. Biotic elicitors are derived from biological sources, such as living bacteria, yeasts, fungal polysaccharides, lipids, and glycoproteins, as well as intracellular molecules, proteins, salicylic acid (SA), jasmonic acid (JA), methyl jasmonate (MeJA), and components of microbial or plant cell walls, such as chitosan (CS) and chitin [1,63].

Studies on the signaling, gene expression, and enzyme activity involved in the manufacture of significant medicinal molecules like SA, paclitaxel, resveratrol, etc. have become more and more prevalent. These are the stages of the focused metabolic engineering process that increase the accumulation of therapeutic SMs [64–66]. Crucial variables affecting the successful generation of biomass and the accumulation of SMs include elicitor concentrations, exposure time, and the age or phase of the culture at the point of elicitor administration [67].

The outcomes of α-NAA treatment along with different spectral lights were conducted by Ali *et al*. [68] to estimate the growth, secondary metabolic activity, and phenolic acid biosynthesis in *A. bracteosa* AR cultures and found out highest AR induction frequency which was 88% and biomass formation (72-g/l FW and 22-g/l DW) were seen in explants incubated with 1.5-mg/l NAA under yellow light, maximum production of polyphenols (TPC;44.2 mg) and flavonoids (TFC;2.51 mg) were found in the AR cultures grown in response to blue light. The highest total protein content (401.6 µg) was reported in the AR grown in the presence of normal white light, and blue spectral light brought about maximum superoxide dismutase (SOD; 2.5 nM) and peroxidase activity (POD;0.85 nM), respectively [68]. Jeet *et al*. [69] studied the effects of CS, MeJA, SA, KCl, NaCl, and polyethylene glycol on indole alkaloids accumulation in callus culture of *Alstonia scholaris* [69]. Addition of yeast extract and CS in liquid hairy root culture of *Astragalus membranaceus* resulted in the elicitation of astragaloside and isoflavones accumulation, respectively [70]. *Corylus avellana* cell suspension culture was exposed to cell extract and culture filtrate derived from *Camarosporomyces flavigenus* fungus for paclitaxel accumulation [71].

NPs have the potential to take the place of biotic elicitors in the elicitation of secondary metabolism. Designing a particular form of monodispersed and stimulus-responsive NPs based on the target metabolite/type of plant cell growth is possible owing to their adaptable physicochemical properties [11]. Also, NPs are frequently utilized as nanocarriers of different biotic and abiotic elicitors to promote the synthesis of specific metabolites *in vitro,* in addition to being used as nano-elicitors [19].

Distinct features of NPs, like their chemical reactivity, binding properties, optical activity, and morphology, make them vary from their corresponding bulk materials. NPs will also have more enhanced properties owing to their size, shape, and structures [72]. Due to their special qualities—like their small size, high surface-to-volume ratio, capacity to engineer electron exchange, and high surface reactive capabilities—NPs readily penetrate and interact with plant cells and tissues' constituent components [5]. Basic methods for intact plants to uptake NPs are foliar absorption either through the stomata or stratum corneum pores of the leaves and root uptake through primary roots, root cell wall pores, or damaged areas from soil [73]. It can also enter through other aerial parts like hydathodes, cuticles, trichomes, or damaged tissues. *In vitro* cultured cells, organs, or plantlets, uptake NPs through cell pores from the NPs-treated medium [74]. The length and width of the stomata and stomatal pores are usually in micrometer scale. Therefore, entry of NPs through stomatal pores is relatively easy and, therefore, is the primary way of foliar uptake. But due to the smaller radii of cuticular pores with approximately 2.4 nm, only smaller NPs pass through cuticles. But NPs of about 50 nm have also been found to enter through cuticles [75]. In case of root uptake, various studies proved that in different plant species, the size exclusion limit varies and, therefore, NPs varying from 3.5 to 140 nm or above can be entered through this pathway in different species [76]. It has been found that in certain instances, NPs larger than the plant cell wall pores were also observed to enter the cells, either by enlarging pre-existing plant cell wall pores or by stimulating the formation of new, larger plant cell wall pores [5,77]. There are many factors influencing the uptake of NPs, which include type, morphology, and size of the NPs; species, growth stage, and condition of plants; and phyllospheric, as well as rhizospheric factors like root exudates and microorganisms, which play a major role in changing morphological properties of NPs and their dissolution and transformation [76].

NPs reach the plant cell membrane after getting internalized through leaves, roots, or cell wall pores. It can be further internalized from the cell membrane either by apoplastic or symplastic pathways. By the apoplastic pathway, NPs can pass through the intercellular spaces between the cell wall and cell membrane of a cell to other cells from the epidermal to the cortex region, but not through the endodermis because of the presence of Casparian strips. Some NPs can penetrate the cell membrane and reach the cytoplasm either by endocytosis, certain membrane-bound transporter proteins, or by inducing new pores utilizing ion carrier molecules and thus enter the symplastic pathway and pass through plasmodesmatal connections between adjacent cells till vascular bundles from where they will be transported to the entire length of plants through xylem and phloem [78,79]. Thus, entered NPs can interact with intracellular organelles and elements and can cause stress and accumulation of reactive oxygen species (ROS), which may interrupt both primary and secondary metabolic pathways of plants and thus can lead to elicitation of SMs [5].

5. NANOMATERIALS AS ELICITORS OF PLANT SECONDARY METABOLITES

Nanomaterials are classified into three main sub-classes: (1) metallic NPs (MNPs), (2) metal oxide NPs (MONPs), and (3) carbon-related nanomaterials for elicitation of plant SMs [5]. Several nanomaterials have been studied for their effect on various plant species and cultures in stimulating the secondary metabolism and thus the production of important SMs, as listed in Table 2.

5.1. Metallic Nanoparticles

Different MNPs (Ag, Cu, Au, Co, Zn, Cu–Au bimetallic, etc.) have been used as plant SM elicitors in a variety of species due to their distinctive features. MNPs have been extensively exploited in a variety of plant species, and reports of their effects on SM production, genetic modification, mass propagation, and microbial content eradication have been obtained [5]. According to the reports, the features of the MNPs (i.e., the concentration employed, the exposure period, their size, and their synthetic origin), as well as the nature of plant culture, had an effect on SM production [1,80]. Kruszka *et al*. [81] reported that MNPs induced more effect on secondary metabolism in plants compared to MONPs.

Within *in vitro* cultures of different plant species, Ag NPs are unquestionably the most utilized MNPs as elicitors of plant SM

Table 2. Few examples of SMs elicitation using NPs as elicitors in PTC.

NPs	Synthesis method	Effective concentration	Plant sp.	Culture type	SMs	Reference
Ag	Biogenic	50 mg l^{-1}	*Capsicum frutescens*	Cell suspension culture	Capsaicin	[82]
Ag	Not specified	0.625 mg l^{-1}	*Aloe vera*	Cell suspension culture	Aloin	[83]
Ag	Chemical	100 mg l^{-1}	*Hyoscyamus muticus*	Hairy root culture	Hyoscyamine, Scopolamine	[84]
Ag	Chemical	1 mg l^{-1}, 2 mg l^{-1}	*Catharanthus roseus*	Callus culture	Vindoline, Vincristine, Catharanthine, Vinblastine	[85]
Ag	Chemical	0.09 mg l^{-1}	*Caralluma tuberculata*	Callus culture	Phenolics, Flavonoids	[39]
Ag	Biogenic	0.125 mg l^{-1}, 0.0625 mg l^{-1}	*Fagonia indica*	Callus culture	TPC, TFC	[13]
Ag	Biogenic	5 mg l^{-1}	*Momordica charantia*	Cell suspension culture	Flavonols, Hydroxybenzoic acids, Hydroxycinnamic acids	[86]
Ag	Chemical	45 mg l^{-1}	*Stevia rebaudiana*	Callus culture	Stevioside	[87]
Co	Chemical	5 mg l^{-1}	*Artemisia annua*	Cell suspension culture	Artemisinin	[88]
Se	Biogenic	0.1 mg l^{-1}	*Caralluma tuberculata*	Callus culture	TPC, TFC	[89]
Se	Chemical	10 mg l^{-1}	*Momordica charantia*	Seed culture	Soluble phenols	[90]
Ag	Chemical	0–50 mg l^{-1}	*Hypericum perforatum*	Cell suspension culture	Bisxanthone, Gancaonin O, Fusaroskyrin	[81]
Au					Hyperxanthone C	
Cu					Apigenin	
Pd					Emodin	
CuO	Chemical	50 mg l^{-1}	*Lavandula stoechas*	Seedlings	Lavandulol, Germacrene D, 1,8-cineole, and (E)-nerolidol	[91]
CuO	Chemical	10 mg l^{-1}	*Ocimum basilicum*	Callus culture	TPC, TFC, Rosmarinic acid, Chicoric acid and Eugenol	[92]
CuO	Chemical	20 mg l^{-1}	*Stevia rebaudiana*	*In-vitro* regenerants	Rebaudioside A, Stevioside	[93]
CuO	Biogenic	0.5 mg l^{-1}	*Vigna radiata*	Callus culture	Glycosides, Phenolics	[94]
CuO	Chemical	10 mg l^{-1}	*Stevia rebaudiana*	Shoot organogenesis	Steviol glycosides, TPC, TFC	[95]
CuO	Chemical	10 mg l^{-1}, 100 mg l^{-1}	*Stevia rebaudiana*	Callus culture	TPC, TFC	[96]
CuO	Biogenic & Chemical	20 mg l^{-1}	*Papaver orientale*	Cell suspension culture	Morphine, Thebaine, Codeine	[97]
Fe_3O_4	Chemical	50 mg l^{-1}	*Artemisia annua*	Seedling culture	Artemisinin	[98]
Fe_3O_4	Chemical	75 mg l^{-1}	*Dracocephalum kotschyi*	Hairy root culture	TPC, TFC, Rosmarinic acid, Xanthomicrol, Cirsimaritin and Isokaempferide	[99]
MnO	Chemical	25 mg l^{-1}	*Ocimum basilicum*	Callus culture	TFC	[92]
SiO_2	Chemical	100 mg l^{-1}	*Dracocephalum kotschyi*	Hairy root culture	Rosmarinic acid, Xanthomicrol, Cirsimaritin and Isokaempferide	[100]
TiO_2	Chemical	2 mg l^{-1}	*Catharanthus roseus*	Callus culture	Vindoline, Vincristine, Catharanthine, Vinblastine	[85]
TiO_2	Chemical	4.5 mg l^{-1}, 6 mg l^{-1}	*Cicer arietinum*	Embryo callus culture	Gallic acid, Chlorogenic acid, Cinnamic acid, o-Coumaric acid, Tannic acid	[101]
TiO_2	Not specified	120 mg l^{-1}	*Aloe vera*	Cell suspension culture	Aloin	[83]
ZnO	Chemical	1 mg l^{-1}	*Bacopa monnieri*	Cell suspension culture	Bacoside A	[102]
ZnO	Biogenic	0.1 mg l^{-1}	*Linum usitatissimum*	Cell suspension culture	TPC, TFC, Lignans. Neolignans	[103]
ZnO	Chemical	2 mg l^{-1}	*Stevia rebaudiana*	*In-vitro* regenerants	Rebaudioside A, Stevioside	[93]

Continued

NPs	Synthesis method	Effective concentration	Plant sp.	Culture type	SMs	Reference
ZnO	Biogenic	0.5 mg l^{-1}	*Vigna radiata*	Callus culture	Glycosides, Phenolics	[94]
ZnO	Chemical	1 mg l^{-1}	*Stevia rebaudiana*	Shoot organogenesis	Steviol glycosides, TPC, TFC	[95]
ZnO	Chemical	100 mg l^{-1}	*Stevia rebaudiana*	Callus culture	TPC, TFC	[96]
ZnO	Chemical	100 mg l^{-1}	*Ceropegia bulbosa*	Cell suspension culture	Cerpegin	[104]
CeO_2	Chemical	0–50 mg l^{-1}	*Hypericum perforatum*	Cell suspension culture	Emodin anthrone	[81]
CuO					Dihydroxydimethoxyxanthone I	
TiO_2					Quercetin	
ZnO					Gallic acid	
Urea doped-Ag	Biogenic	6 mg l^{-1}	*Triticum aestivum*	Seed culture	TPC and TFC	[105]
Ag- Au	Not specified	1:3 (0.03 mg l^{-1})	*Prunella vulgaris*	Callus culture	TPC and TFC	[106]
Ag-Au	Chemical	3:1 (0.03 mg l^{-1})	*Prunella vulgaris*	Cell suspension culture	TPC, TFC	[51]
Ag-Cu	Biogenic	3:1 (0.03 mg l^{-1})	*Artemisia absinthium*	Callus culture	TPC	[107]
Ag-Cu	Biogenic	1:3 (0.03 mg l^{-1})	*Artemisia absinthium*	Callus culture	TFC	[107]
Au-Cu	Chemical	1:3 (0.03 mg l^{-1})	*Stevia rebaudiana*	AR culture	TPC, TFC	[108]
CS-ZnO	Chemical	1 mg l^{-1}	*Capsicum annuum*	Seed culture	Soluble phenols and Alkaloids	[109]
Graphene oxide	Biogenic	6 mg l^{-1}	*Stevia rebaudiana*	*In-vitro* plants	Stevioside, Rebaudioside A	[110]
MWCNTs	Chemical	100 mg l^{-1}	*Satureja khuzestanica*	Callus culture	Rosmarinic acid, Caffeic acid, several phenolics and flavonoids	[111]
MWCNTs	Chemical	250 mg l^{-1}	*Taxus baccata*	Cell suspension culture	Paclitaxel	[112]
MWCNTs	Chemical	250 mg l^{-1}	*Satureja khuzistanica*	Nodal segment culture	Rosmarinic acid	[113]
Nano- CS	Chemical	1 mg l^{-1}	*Capsicum annuum*	Seed culture	Soluble phenols, Alkaloids	[114]
Nanosheets	Not specified	10 mg l^{-1}	*Curcuma alismatifolia*	*In-vitro* plantlets	TPC	[115]
SWCNTs	Chemical	50 mg l^{-1}	*Thymus daenensis*	Callus culture	Rosmarinic acid, *trans*-ferulic acid, Catechin, Hesperidin, Vanillin and Carvacrol	[116]

NPs: Nanoparticles; SMs: Secondary metabolites; TPC: Total phenolic content; TFC: Total flavonoid content; MWCNTs: Multi-walled carbon nanotubes; SWCNTs: Single-walled carbon nanotubes. All values are reported as per original studies. Units were standardized to mg l^{-1}.

[117,118]. A variety of plant species in various production systems have been successfully elicited by Ag NPs [5]. Ag NPs (3.0 mg l^{-1})-treated *Capsicum frutescens* cell suspension culture produced two-times more capsaicin [82]. Khan *et al*. [105] studied *Alnus nitida* leaves extract-synthesized Ag NPs and urea-doped Ag NPs (U-Ag NPs) and their effects on the elicitation of SM production in wheat seeds under *in vitro* conditions . Results showed that U-Ag NPs have the potential to be a significant elicitor of secondary metabolism in comparison with Ag NPs. Wheat seed cultures treated with U-Ag NPs showed increased production of phenolics, flavonoids, and chlorophyll content, as well as antioxidant activity. Callus culture of plants like *C. tuberculata* and *Fagonia indica* treated with Ag NPs has improved the accumulation of total phenolics and flavonoids [13,39]. Synthesis of Aloin, which is primarily used for its medicinal and cosmetic purposes, was dramatically increased by 127% in *Aloe vera* cell suspension treated with Ag NPs (at 0.625 mg l^{-1}) [83]. Hyoscyamine and scopolamine production was enhanced by elicitation using Ag NPs in hairy root culture of *Hyoscyamus muticus* [84]. Treatment with 1 and 2 mg/l of Ag NPs in *C. roseus* callus culture increased the production of vindoline, vincristine, catharanthine, and vinblastine [85]. Chung *et al*. [86] studied the effect of Ag NPs in *Momordica charantia* suspension culture and found that it enhanced the production of flavonols, hydroxybenzoic acids, and hydroxycinnamic acids. *Stevia rebaudiana* callus elicited with 45-mg/l Ag NPs increased the production of stevioside [87]. Gallic acid, coumarin, tannic acid, hesperidin, quercetin, and rutin were synthesized in higher quantities than in the control when *Juniperus procera* callus culture was elicited with Ag NPs [119].

Kruszka *et al*. [81] studied the effects of Ag, Au, Cu, and Pd NPs treatment on the plant secondary metabolism in cell suspension culture of *Hypericum perforatum* L. and found out that Ag NPs enhanced the accumulation of bisxanthone (540.3-fold), gancaonin O (214.2-fold), fusaroskyrin (98.6-fold), 2-deprenyl-7-hydroxyrheediaxanthone (38.4-fold), hyper xanthone D (60.8-fold), gemi xanthone A (34.4-fold), trihydroxy xanthone II (98.3-fold), tetrahydroxy methoxyxanthone (77.5-fold), trihydroxy methoxy xanthone I (74.0-fold) and trihydroxydimethoxy xanthone (42.9-fold); Au NPs enhanced the accumulation of hyperxanthone C (96.4-fold), dihydroxydimethoxy xanthone III (82.6-fold), γ-mangostin (81.1-fold), hyperxanthone E

(26.0-fold), bijaponica xanthone C (20.4-fold) and maclurin (10.8-fold); Cu NPs enhanced the accumulation of apigenin (13.7-fold), hyperxanthone A (10.4-fold), kaempferol (7.4-fold), methoxyemodin (7.0-fold), 6-deoxyisojacareubin (6.3-fold) and rhein (5.6-fold); Pd NPs increased the accumulation of emodin (6.9-fold), dulciol E (5.3-fold), garcinone b (4.3-fold), and chrysoobtusin (3.4-fold) [81].

Se NPs were used as an elicitor in callus culture of *C. tuberculata* to elicit total phenolics and flavonoids by Ali *et al*. [89]. Rajaee Behbahani *et al*. [90] elicited soluble phenols in *M. charantia* seed culture by Se NPs. 5 mg/l of Co NPs in *A. annua* suspension culture enhanced the accumulation of artemisinin by 2.25-fold [88].

It has also been shown that bimetallic NPs and other MNP combinations are efficient elicitors of SMs production in a variety of plant species [106,108]. Studies regarding the effect of Ag and Au NPs in combinations (1 : 2, 1 : 3, 2 : 1, and 3 : 1) or individually on callus cultures of *P. vulgaris* L., showed that the application of a 1 : 3 ratio of Ag : Au NPs considerably boosted the total flavonoids (4%) and phenolics (23%) productions [106]. Fazal *et al*. used a 3:1 ratio of Ag and Au NPs, respectively, in *P. vulgaris* suspension culture and enhanced the accumulation of TPC and TFC [51]. Cu–Au bimetallic NPs (in a 3:1 ratio) are effective elicitors to produce greater levels of total phenolic (54%) and flavonoid (20%) contents in an AR culture of *S. rebaudiana* [108]. *Artemisia absinthium* callus was studied for the effect of various ratios of Ag and Cu NPs on the synthesis of SMs and revealed that a 3:1 ratio enhanced total phenolic content and a 1:3 ratio enhanced total flavonoid content [107].

5.2. Metal Oxide Nanoparticles

The literature offers a variety of frequently contradicting findings regarding how plants react when exposed to various MONPs. On the elicitation behavior of several MONPs under *in vitro* cultures of various plant species, numerous investigations have been conducted. Copper oxide (CuO), zinc oxide (ZnO), titanium dioxide (TiO_2), cerium oxide (CeO_2), cadmium oxide, iron oxide (Fe_3O_4), and aluminum oxide NPs are the most often employed MONPs as elicitors of SMs [5].

Fe_3O_4 NPs are easily available for a variety of scientific applications, including the possibility of being used as elicitors of plant SM production due to their straightforward and affordable manufacture [120]. Ayoobi *et al.* [98] studied the effect of Fe_3O_4 NPs in *A. annua* seedlings, and the results revealed that applying Fe_3O_4 NPs was a successful elicitor treatment that improved the generation of artemisinin by 98.5% compared to the control. With *H. perforatum*, researchers were able to produce an intriguing example of the usage of Fe_3O_4 and ZnO NPs, which enhances the plant's accumulation of specific SMs, such as hypericin and hyperforin [121]. Fe_3O_4 NPs-treated hairy root culture of *Dracocephalum kotschyi* produced 9.7, 11.87, 3.85, and 2.27-fold more amount of rosmarinic acid, xanthomicrol, cirsimaritin, and isokaempferide, respectively [99].

Due to their widespread use in the role of a UV filter in sunscreens, TiO_2 NPs are among the most widely dispersed NPs in the environment. As a result, plants are more vulnerable to directly absorbing TiO_2 NPs from the environment [122]. Recent research revealed that ionic Ti is the main trigger for the physiological impacts of TiO_2 NPs on plants [123]. In addition, numerous research teams have examined the ability of TiO_2 NPs to stimulate the production of plant SMs utilizing both *in vivo* and *in vitro* conditions [101,124]. Treatment with Ag NPs (2 mg/l) in *C. roseus* callus culture increased the production of vindoline, vincristine, catharanthine, and vinblastine [85]. Aloin concentration in *A. vera* cell suspension cultures treated with TiO_2 NPs increased to 118% using *in vitro* systems [83]. Similar to this, when subjected to various concentrations of TiO_2 NPs, the phenolic and flavonoid contents in *Cicer arietinum* callus culture increased [101].

Kruszka *et al*. [81] studied the effects on the plant secondary metabolism in cell suspension culture of *H. perforatum* L. treated with CuO, ZnO, CeO_2, and TiO_2 NPs. They found out that the CuO NPs increased the production of dihydroxydimethoxyxanthone I (2.7-fold), hyperxanthone B (2.4-fold), and trihydroxyxanthone I (2.4-fold); CeO_2 NPs enhanced the accumulation of emodin anthrone 4.7-fold; ZnO NPs increased the accumulation of procyanidin B and gallic acid 11.3 and 2.0-fold, respectively; and TiO_2 NPs increased quercetin production 2.5-fold [81]. Javed *et al*. [96] developed a callus culture of *S. rebaudiana* and treated it with different concentrations of ZnO and CuO NPs. Phytochemical analysis of callus culture treated with ZnO NPs showed the highest TPC of 5.65 µg/mg of DW and the highest TFC of 2.85 µg/mg of DW when exposed to 100 mg/l of ZnO NPs. Phytochemical analysis of callus culture treated with CuO NPs showed the highest TPC of 5.88 µg/mg of DW when exposed to 10 mg/l of CuO NPs and the highest TFC of 2.23 µg/mg of DW when exposed to 100 mg/l of CuO NPs [96].

Treatment with 2 mg/l of ZnO and 20 mg/l of CuO NPs in the *in vitro* regenerants of *S. rebaudiana* led to an increase in rebaudioside A and stevioside content by 4.42 and 1.28% [93], and with 1-mg/l ZnO and 10-mg/l CuO NPs [95] increased the production of steviol glycosides, TPC, and TFC. Iqbal *et al*. studied the effect of ZnO and CuO NPs (0.5 mg/l) in *Vigna radiata* callus and reported an enhanced production of glycosides and phenolics [94]. ZnO and CuO NPs treated *S. rebaudiana* callus culture showed an increase in TPC and TFC [95]. Bacoside A production was enhanced by two-fold on treating *Bacopa monnieri* cell suspension with ZnO NPs [102]. Asadollahei *et al*. [91] enhanced the production of lavandulol, germacrene D, 1,8-cineole, and (E)-nerolidol by 21.68%, 17.21%, 9.33%, and 8.11%, respectively [91]. ZnO NPs were used to elicit TPC, TFC, lignans, and neolignans in suspension culture of *Linum usitatissimum* [103]. Kashyap *et al*. [104] enhanced the production of cerpegin by 100-mg/l ZnO NPs in *Ceropegia bulbosa* suspension culture. *Papaver orientale* cell suspension was treated with CuO NPs, which increased the accumulation of morphine, thebaine, and codeine [97]. Total phenolics, flavonoids, rosmarinic acid, chicoric acid, and eugenol content were increased in callus culture of *O. basilicum* using 10-mg/l CuO NPs and 25-mg/l MnO NPs as elicitors [92]. SiO_2 NPs-treated hairy root culture of *D. kotschyi* produced 8.26, 13, 13.42, and 10-fold more amount of rosmarinic acid, xanthomicrol, cirsimaritin, and isokaempferide, respectively [100].

5.3. Carbon-Related Nanomaterials

Carbon-based nanomaterials are promising nanomaterials for application in medicine, agriculture, and plant biology. The extensive application of such nanomaterials in biological fields is due to their unmatched optical, electrical, mechanical, and thermal properties [125]. It includes single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), CS NPs, derivatives of fullerene (Buckminsterfullerene or Buckyball), nanosheets, nano-cones, nano-horns, nano-diamonds, nano-fibers, nano-onions, nano-beads, graphene, and carbon dots [126,127,116,115].

Samadi *et al*. [116] studied the effect of SWCNTs in callus culture of *Thymus daenensis* and found that supplementing the MS media with 50-mg/l SWCNTs can increase the production of several valuable phenolics and flavonoids like rosmarinic acid, *trans*-ferulic acid, catechin, hesperidin, vanillin, and carvacrol. *Satureja khuzestanica in vitro* callus cultures were used to study the inductive effect of MWCNTs

on the production of SMs. The optimal concentration of MWCNTs used was 100 mg/l, and this increased the biomass as well as the accumulation of total phenolics (12%), flavonoids (3%), rosmarinic acid (12.32 mg/g DW), and caffeic acid (9.2 mg/g DW) contents [111]. Fatemi *et al*. [113] investigated the impact of MWCNTs (250 mg/l) on nodal segment culture of *Satureja khuzistanica* and found that MWCNTs exposure significantly increased the accumulation of rosmarinic acid. Asgharzadeh *et al*. [112] studied the effect of MWCNTs in enhancing paclitaxel production in the cell suspension culture of *Taxus baccata*. Their results showed that 100- and 250-mg/l MWCNT treatment led to secretion of 18% and 96% of total paclitaxel content into the culture medium, respectively, as well as a 2.7-fold and 6.7-fold increase in paclitaxel production, respectively [112].

CS is frequently utilized in biomedicine and agriculture. It has desirable, distinctive qualities, including non-toxicity and biodegradability. In the seed culture of *Capsicum annuum*, 1 mg/l of nano-CS was utilized as an effective elicitor of soluble phenols and [114]. Asgari-Targhi *et al*. [109] synthesized CS-encapsulated ZnO nanocomposite (CS-ZnO NP), and *C. annuum* seed culture was treated with it and assessed its efficiency to produce SMs. It was found that CS-ZnO NPs treatment significantly enhanced secondary metabolism and thereby increased the accumulation of soluble phenols by 40% and alkaloids by 60.5% [109].

Curcuma alismatifolia in vitro plantlets were evaluated for the effects of various concentrations of nanosheets combined with JA and SA, and revealed a positive influence on the accumulation of total phenolics [115]. Graphene oxide NPs were used for the elicitation of stevioside and rebaudioside A content in *in-vitro* plantlets of *S. rebaudiana* [110].

6. MECHANISM OF NANOPARTICLE-MEDIATED ELICITATION OF SECONDARY METABOLITES

Elicitation mechanisms can involve thousands of mediators from numerous signaling channels, and their interactions make them extremely complicated. The origin, specificity, exposure type, timing, and concentration of the elicitors, as well as plant-dependent factors like the stage of development, cellular cycle, type of tissue, nutritional conditions, elicitor uptake by media/soil/aerial, physicochemical environment, and many other factors, affect how these events change [5]. Therefore, it is very difficult to suggest an all-encompassing model for the mechanism by which nanomaterials elicit the synthesis of plant SMs. The scheme of the general mechanism of the elicitation mechanism in plant secondary metabolism is depicted in the Figure 3.

A chain of events gets started as soon as the NPs reaches inside the cell. There are numerous receptors on the cell surface and membranes, and multiple elicitor binding sites have been discovered [74]. The initial event is the receptor–elicitor contact, where the NPs that are being applied as the elicitor act as the primary messenger molecule. The interaction of NPs with the transmembrane receptor-like kinases (RLKs) results in a signal transduction cascade. The binding of NPs with RLKs causes Ca^{2+} influx from intracellular stores [5]. Release of Ca^{2+} from apoplastic space also occurs, which adds to the Ca^{2+} level in the cytosol [128]. Elicitor contact with specific receptors will also lead to plasma membrane depolarization, which will further lead to the activation of certain ion channels in the plasma membrane to result in the efflux of Na^{+}, K^{+}, and Cl^{-} and H^{+} influx. This will further increase the influx of Ca^{+} and deactivation of ATPase, leading to cytoplasmic acidification and extracellular alkalinization [74,129]. The interaction of NPs with G protein-coupled receptors will activate the G-protein and further initiate the activation of phospholipase-C (PLC) on the membrane [130]. PLC then hydrolyzes phosphatidylinositol 4,5-bisphosphate and yields two secondary messenger molecules inositol triphosphate (IP_3) and diacylglycerol. IP_3 then diffuses into the cytosol and leads to the release of Ca^{2+} from intracellular calcium reservoirs through Ca^{2+}-gated ion channels [131].

The released Ca^{2+} also regulates the PLC, which will further increase the Ca^{2+} concentration within the cytoplasm and also the production of other

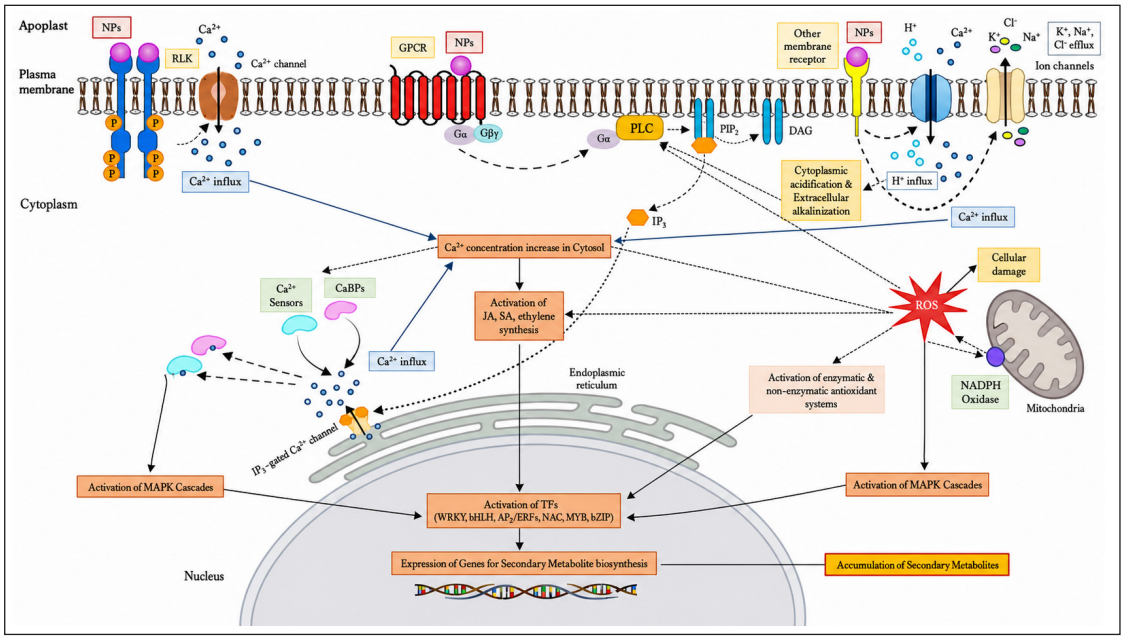

Figure 3. Mechanism of NP-mediated elicitation of SMs. NPs: Nanoparticles, RLK: Receptor-like kinase; GPCR: G protein-coupled receptor, PLC: Phospholipase-C, PIP: Phosphatidylinositol 4,5-bisphosphate, IP3: Inositol 1,4,5-triphosphate, DAG: Diacylglycerol, CalPs: Calcium-binding proteins, JA: Jasmonic acid, SA: Salicylic acid, MAPK: Mitogen-activated protein kinases, TFs: Transcription factors, ROS: Reactive oxygen species.

secondary messengers like JA, SA, ethylene, etc., which can directly or indirectly modulate secondary metabolism [132]. It is also known that Ca^{2+} activates several oxidases, like cell membrane NADPH oxidase, apoplastic POD, and several other oxidases in cellular organelles like chloroplasts, mitochondria, and peroxisomes. The activation of these oxidases will lead to oxidative burst by which enormous amounts of ROS are generated within the cell [129]. It includes hydrogen peroxide, superoxide anions, hydroxyl radicals, and singlet oxygen. NPs interfere with the electron transport chain in chloroplasts and mitochondria, which also leads to the generation of ROS and reactive nitrogen species and nitric oxide [128,129]. These are known to cause cellular damage by protein modification, lipid peroxidation, and DNA damage. These are also known to activate certain enzymatic and non-enzymatic antioxidant systems in plants like SOD, ascorbate peroxidase, catalase, and glutathione-S-transferase, which help to convert ROS and detoxify them. Based on the balance between generation and scavenging of ROS within the cell, they either cause oxidative damage or act as signaling molecules [128]. ROS also causes damage to the plasma membrane and activation of PLC, which will further lead to a Ca^{2+} spike [133]. ROS is also known to activate another cascade called the mitogen-activated protein kinase (MAPK) cascade, and also the production of jasmonates, ethylene, etc. NPs' perception by membrane receptors may also activate the MAPK cascade. All these will lead to an increase in Ca^{2+} concentration approximately within 5 minutes of NPs administration, which marks a very early response [129,130].

The increased Ca^{2+} level within the cytoplasm can be sensed by certain calcium-binding proteins or calcium sensors like calmodulin, calmodulin-like proteins, phospholipase-D, annexins, calreticulin, calnexin, and pistil-expressed calcium binding proteins or by calcium-dependent protein kinases. These Ca^{2+} signaling will further transfer the information downstream to initiate a cascade of phosphorylations, which includes activation of MAPK cascades, which will lead to regulation of expression of genes involved in secondary metabolism mainly by altering the phosphorylation status of certain transcription factors (TFs) of target genes [128].

The complex signaling pathways that occur on exposure to the NPs, like MAPK cascades, Ca^{2+}, ROS, JA, SA, and ethylene signaling, and the cross-talks between these, together activate several TFs that will bind to the target genes and regulate the defense mechanism, thereby enhancing SM production [134]. The major TFs involved in secondary metabolism are: i) MYB TF, which contains the MYB DNA-binding domain. It is involved in the biosynthetic pathways of anthocyanin, proanthocyanidins, glucosinolate, phenylpropanoids, flavonoids, and hydroxycinnamic acid amide (HCCAs). ii) bHLH TFs, which are found to act in combination with MYB TF in the regulation of anthocyanin production. bHLH TF-like MYC2 plays a major role in terpenoid indole alkaloid (TIA), nicotine, anthocyanin, alkaloids, glucosinolate, diterpenoid, phytoalexin, and saponin biosynthesis. iii) AP2/ERF family TFs have a DNA-binding AP2 domain, such as in the ORCA protein, which has a major role in controlling multiple genes involved in TIA biosynthesis. It is also found to be involved in artemisinin, artemisinic acid, nicotine, vinblastine, bisindole alkaloids, steroidal glycoalkaloids, saponin, and lignin biosynthesis. iv) WRKY family TFs are found to have a role in biosynthesis of gossypol, artemisinin, volatile terpene, camalexin, phytoalexin, HCCA, resveratrol, benzylisoquinoline alkaloid, taxol, TIA, etc. v) NAC TFs are found to be involved in camalexin and putrescine biosynthesis. vi) bZIP TFs have major roles in lignin, phenolic acids, flavonoids, terpenoids, alkaloids, anthocyanin, tanshinone, and artemisinin biosynthetic pathways [6,135,136].

7. CONCLUSION AND FUTURE PROSPECT

Since there is a very high demand for SMs in various fields and because of the improved knowledge about commercially important plant-based SMs, currently, the use of PTC techniques has increased for their large-scale production. PTC is an advantageous technique in order to synthesize biocompounds from plants that are even on the verge of extinction for their conservation. It also provides a good yield irrespective of any environmental or geographical conditions if sterile and appropriate conditions are properly maintained.

In order to enhance the production potential of important SMs in terms of both quantity and quality in culture systems, the elicitation technique has been proven to be highly advantageous. Use of nanomaterials (MNPs, MONPs, and other carbon-related nanomaterials) due to their specificity and enhanced properties is reported to be a potential approach. Various research studies proved the improved production of diverse plant SMs of commercial importance on elicitation using different types of nanomaterials in various culture conditions. Concentration, size, exposure time, time of addition of nanomaterials, as well as the plant species, type, and age of culture, are the main factors to be given importance during the elicitation process for the maximum result.

Further research by addressing the limitations of present methods, as well as by utilizing the emerging technologies, will definitely help to produce important SMs more sustainably while giving enhanced yield. For this, there is an importance to move forward by acknowledging the current limitations. Some studies have reported certain concentrations of NPs to be toxic to the plants. Therefore, the usage of smaller concentrations of NPs seems to be more efficient in generating higher amounts of SMs without causing phytotoxicity to the plants. Further studies incorporating metabolomics, transcriptomics, and genomics will help provide a wide understanding of the cellular and molecular-level events that take place on elicitor administration in plants. Thus, specific steps in metabolic pathways, specific genes and proteins that experience alterations and are responsible for SM generation can be identified, and thus the elicitation mechanism can be improved accordingly. Moreover, there are still many sources unveiled for green synthesis of safer, less toxic NPs as well as for SM production. More research improving the existing techniques and approaches more sustainably, without compromising efficiency and productivity, has to be introduced to bring out a major impact, generating better economic productivity and environmental sustainability.

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9. AUTHOR'S CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

10. ETHICS APPROVAL

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

14. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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