Review

Nano-Elicitation as an Effective and Emerging Strategy for In Vitro Production of Industrially Important Flavonoids

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Featured Application: Nanoparticles (NPs) emerged as novel and effective elicitors for increasing the production of industrially important flavonoids.

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Abstract: Flavonoids represent a popular class of industrially important bioactive compounds. They possess valuable health-benefiting and disease preventing properties, and therefore they are an important component of the pharmaceutical, nutraceutical, cosmeceutical and medicinal industries. Moreover, flavonoids possess significant antiallergic, antihepatotoxic, anti-inflammatory, antioxidant, antitumor, antiviral, and antibacterial as well as cardio-protective activities. Due to these properties, there is a rise in global demand for flavonoids, forming a significant part of the world market. However, obtaining flavonoids directly from plants has some limitations, such as low quantity, poor extraction, over-exploitation, time consuming process and loss of flora. Henceforth, there is a shift towards the in vitro production of flavonoids using the plant tissue culture technique to achieve better yields in less time. In order to achieve the productivity of flavonoids at an industrially competitive level, elicitation is a useful tool. The elicitation of in vitro cultures induces stressful conditions to plants, activates the plant defense system and enhances the accumulation of secondary metabolites in higher quantities. In this regard, nanoparticles (NPs) have emerged as novel and effective elicitors for enhancing the in vitro production of industrially important flavonoids. Different classes of NPs, including metallic NPs (silver and copper), metallic oxide NPs (copper oxide, iron oxide, zinc oxide, silicon dioxide) and carbon nanotubes, are widely reported as nano-elicitors of flavonoids discussed herein. Lastly, the mechanisms of NPs as well as knowledge gaps in the area of the nano-elicitation of flavonoids have been highlighted in this review.

Keywords: flavonoids; nanoparticles; elicitation; biotechnology; plant secondary metabolites

1. Introduction

Flavonoids are plant secondary metabolites which have gained importance in the biotechnology industry due to their unique health related properties [1]. They are found in almost all of the growing parts of plants responsible for the pigmentation of fruits, flowers and leaves. Moreover, flavonoids such as anthocyanins, tannins and phytoalexins impart protection to plants against ultraviolet radiation, herbivores and pathogens, respectively [2]. Besides their relevance in plants, flavonoids are also associated with a

vast spectrum of health enhancing effects and are an indispensable component in a variety of pharmaceutical, nutraceutical, cosmetical and medical applications [3,4]. Flavonoids possess significant antiallergic, antihepatotoxic, anti-inflammatory, antioxidant, antitumor, antiviral, antibacterial, antiosteoporosis as well as cardio-protective activity. Antioxidant, antihepatotoxic, anti-inflammatory, antiallergic, antitumor, antiosteoporosis, antiviral, antibacterial and cardio-protective potential have been reported in many flavonoids from various plant species [5–9]. In vivo studies also support the possible use of flavonoid rich plants in treating diseases. Recently, Bergamot juice, which is rich in flavonoid content, was used to treat experimental periodontitis in rats. The results showed that flavonoids present in Bergamot juice reduced the expression of inflammatory markers associated with gingival inflammation, and downregulated NF- κ B activation as well as apoptosis [4]. Therefore, when consumed in the human diet, these compounds promote health and prevent diseases [4,5]. Flavonoids also influence the quality and stability of food as they add flavor and color to food and act as a food preservative [10]. These properties make flavonoids an important commercial biomolecule, which has led to attempts for the in vitro production of flavonoids via the plant tissue culturing technique.

Plants are capable of synthesizing an overwhelming variety of low molecular weight organic compounds, called plant secondary metabolites or plant specialized metabolites, which help plants to colonize diverse and challenging environments [11]. Currently, about 100,000 such compounds have been isolated from plants classified into major groups, which include terpenes, phenolics and alkaloids [12]. Flavonoids form a part of a large group of phenolic compounds and an essential component of plant cells [13]. Phenolic compounds have one or more hydroxyl groups attached directly to an aromatic ring, while their subgroup, flavonoids, is characterized by the presence of 15 carbon atoms in the flavonoids' basic skeleton containing two aromatic rings. The carbon atoms in flavonoids are arranged in C₆-C₃-C₆ form, which corresponds to two aromatic compounds linked together via three carbon atoms [14,15]. The arrangement of the C₃ group determines the classification of flavonoids, giving rise to three classes: flavonoids, isoflavonoids and neoflavonoids [16] (Figure 1). The biosynthesis of flavonoids is initiated by the condensation of one *p*-coumaroyl-CoA molecule with three molecules of malonyl-CoA in the presence of chalcone synthase to form chalcone. Chalcone is isomerized by an enzyme chalcone isomerase, which converts chalcones to flavanones. From these intermediate compounds, the pathway diverges into several side branches, each giving rise to a different class of flavonoids [17].

Figure 1. Flavonoids' structures and classification.

Recently, medicinal plants have been exploited for flavonoids to treat human maladies naturally [18]. With the rise in industrial demand for bioactive compounds from medicinal plants, there is a shift towards the use of in vitro culture systems for the mass production of secondary metabolites in a shorter time. The in vitro culturing technique avoids the destruction of plants' natural habitat, cutting down plant roots or underground parts and the over-exploitation of wild species [19]. Most prominently, callus and cell suspension cultures of several plants have been established for the enriched production of flavonoids. Both culture types ensure homogeneity in in vitro cell culture, a higher growth rate and productivity, as well as good reproducibility [20]. In addition to this, transgenic hairy root cell lines for the stable production of flavonoids have also been established [21]. Despite the successful in vitro production of plant secondary metabolites, there are still some bottlenecks, such as inconsistent yield, poor quality and lower productivity for large scale cultivation. To counter this, biotechnologists have devised different strategies to enhance the production of secondary metabolites in in vitro cultures, such as media modification, genetic engineering, precursor feeding, screening of high yielding cell lines, bioreactors, immobilization, biotransformation and elicitation [22].

Among different strategies, elicitation is the best known and effective yield enhancement strategy. Elicitation is the addition of exogenous elicitors, which are compounds that stimulate any type of plant defense and promote plant secondary metabolism to impart protection to the plant against stress [23]. Elicitors are perceived by specific receptors/ion channels in the plasma membrane, trigger gene expression and induce a stress response in plants, which may result in the production of higher amounts or new forms of valuable secondary metabolites [24]. To date, different types of biotic and abiotic elicitors have been used for the elicitation of plant secondary metabolism. Among different elicitors, nanoparticles (NPs) showed a great potential to act as novel elicitors of flavonoids in plants due to their unique properties. Currently, different types of NPs have been used as novel and effective elicitors of flavonoids in in vitro cultures of various plant species, as shown in Figure 2 [25]. Different types of NPs, including metallic NPs, metal oxide NPs and carbon-related NPs, are widely reported as novel elicitors of flavonoids. These NPs include silver NPs (AgNPs), copper NPs (CuNPs), iron oxide NPs (Fe_3O_4 NPs), zinc oxide NPs (ZnONPs), copper oxide NPs (CuONPs), carbon nanotubes and chitosan based NPs [26]. In this review, an in-depth literature on the role of NPs as elicitors of industrially important flavonoids has been critically reviewed, along with the mechanism of nano-elicitation. Furthermore, the review is concluded with future directions.

Figure 2. Graphical representation of different types of nano-elicitors of commercially important flavonoids in various in vitro systems of plants (NPs: nanoparticles).

2. Mechanism of Nano-Elicitation

Since little is known about most of plant secondary metabolism, the effects of elicitors on plant cells or tissue cultures are therefore difficult to gauge. The mechanism employed by elicitors is complex and distinctive. Elicitors act as a signal which is received by the elicitor specific receptors located on plasma membranes [27]. Several elicitor-binding sites have been identified in cell plasma membranes for a range of elicitors. Transmembrane receptor-like protein kinases (RLKs) are one of the well-known categories of receptors which might be concerned with the perception of different types of stimuli [28]. NPs can directly bind receptors (e.g., chitosan-NPs) and/or trigger the production of the second endogenous messenger that could bind to these receptors [20].

After the binding of receptor-NPs, the initial response to NPs involves an active exchange of ions, i.e., Cl^- and K^+ efflux and Ca^{2+} influx occur across the cell membrane into the cytosol, as shown in Figure 3. Ca^{2+} influx is an important step during the elicitation process as it further modulates various physiological and cellular pathways. Cytoplasm acidification occurs in response to H^+ -ATPase inactivation and an increase in extracellular pH. Cytoplasmic acidification is important for signal transduction, leading to the oxidative burst and biosynthesis of plant secondary metabolites.

Figure 3. Representation of a proposed mechanism involved in nano-elicitation of commercially important flavonoids in various in vitro systems of plants (Note: dashed arrows indicate indirect reactions and the continuous arrows indicate direct ones; ROS: reactive oxygen species; MAPKs: mitogen-activated protein kinases; NPs: nanoparticles; JA: jasmonate; MeJA: methyl jasmonate; SA: salicylic acid).

These events lead to reversible phosphorylation and dephosphorylation of plasma membrane proteins and cytosolic proteins and the activation of protein kinases (PK) and mitogen-activated protein kinases (MAPK). The involvement of GTP binding proteins (G-proteins) during elicitation is also reported. G-proteins probably respond by

activating phospholipases, leading to the downstream production of plant secondary metabolites [29–31].

The PLC (phospholipase-C)/IP₃-DAG (Inositol trisphosphate- diacylglycerol)/PKC (protein kinase C) cascade also plays a key role during elicitation. The second calcium influx occurs due to PLC activation and inositol triphosphate (IP₃) released Ca²⁺ from intracellular calcium stores, such as the vacuoles, Golgi apparatus, and endoplasmic reticulum [32]. The increase in cytoplasmic calcium levels activates the defense response, either directly by calcium or through calcium sensors, such as calmodulins and Ca²⁺-dependent protein kinases (CDPKs). Increased intracellular Ca²⁺ level activates NADPH oxidase and the subsequent generation of ROS (reactive oxygen species) [20]. ROS induces the expression of many defense genes and secondary metabolite biosynthetic genes, such as phenylalanine ammonia lyase (PAL), and the induction of signaling molecules, such as salicylic acid (SA), ethylene (EA) and jasmonate (JA). These signaling molecules further activate the pathways of secondary metabolite production in plant cells [33].

Several factors such as the concentration of an elicitor, the morphology of an elicitor, exposure time, the type of in vitro culture, nutrient composition, growth regulators and plant species influence the production of secondary metabolites [25].

3. Nano-Elicitation of Flavonoids

In last few years, a variety of nanomaterials have been reported for the elicitation of flavonoids in different in vitro cultures of plant species.

3.1. AgNPs as a Novel Elicitor of Flavonoids

AgNPs find their applications in medicine, biotechnology and agriculture owing to their desirable physico-chemical properties [34]. Amongst different NPs, AgNPs are widely reported as a novel elicitor of flavonoids in in vitro culture (Table 1). *Momordica charantia*, commonly known as bitter melon, is a highly nutritive vegetable containing bioactive compounds such as phenolics and flavonoids [35]. The cell suspension culture of *M. charantia* was elicited with *Bacillus licheniformis* biosynthesized AgNPs (50 nm) at 5 mg/L concentration. The elicited culture showed enhanced concentration of different flavonoids including quercetin, kaempferol, catechin, myricetin, rutin, naringenin and biochanin A [36]. Flavonols are the most ubiquitous flavonoids found in food, also called 3-hydroxylavones. They are closely related to flavones in structure but differ in having a hydroxyl group at 3-position on the C-ring, while flavones contain a ketone group with an unsaturated carbon-carbon bond [37]. Quercetin, being the most abundant flavonol in plants, possesses anti-inflammatory, anti-oxidative and anticancer activities, and it is therefore a widely studied flavonoid. As quercetin has a special ability to scavenge highly reactive species such as hydroxyl radical, so it has importance as a nutraceutical [38]. The hairy root culture of *Cucumis anguria* when elicited with AgNPs showed an increase in the accumulation of phenolic compounds as well as flavonoid compounds. AgNP elicited culture showed a higher amount of flavonols (3136.20 µg/g) as compared to non-elicited culture [39]. Similarly, biogenic AgNPs effectively elicited the hairy root culture of *Brassica rapa* at concentrations 50, 100, and 150 mg/L. Hairy roots treated with 100 mg/L AgNP solution showed a gradual increase in the concentration of total phenolic content (TPC) and total flavonoid content (TFC), while treatment with 150 mg/L decreased TPC and TFC. Moreover, nine different types of flavonoids (myricetin, naringenin, catechin, quercetin, rutin, kaempferol, biochanin A, formononetin, and hesperidin) were accumulated in a higher quantity in AgNP elicited culture [40].

The influence of biologically synthesized polydispersed AgNPs from a pod extract of *Cola nitida* on the phytochemical profile of *Amaranthus caudatus* plant was investigated. There was 32.58, 35.80, and 7.20% improvement in flavonoid content for 25, 50 and 100 ppm concentration of AgNPs, respectively. Moreover, kaempferol and quercetin were the most abundant flavonols produced at 50 ppm AgNP concentration [41]. The callus culture of *Caralluma tuberculata* when elicited with 90 µg/L AgNPs (40 nm) led to 1.8 mg TFC

production [42]. *Caralluma tuberculata* contains valuable bioactive compounds mostly used to treat diabetes, asthma, paralysis, inflammation, obesity and fever [43].

Anthocyanins are another member of the flavonoids family which have angiocardopathy, high antioxidant and cancer prevention properties [44]. Anthocyanins are water soluble colored pigments which have been used as food pigments, nutraceuticals and traditional medicine [45]. When the seedlings of *Arabidopsis thaliana* were elicited with 50 μ M spherical shaped AgNPs, a 56% increase in anthocyanins was observed. Moreover, anthocyanin accumulation was correlated with the increase in the expression of antioxidant enzyme Cu/Zn superoxide dismutase 2 [46].

When the cell suspension culture of *Linum usitatissimum* was elicited with AgNPs during the early phase, enhanced flavonoid production was observed, which declined during the later phase, and the highest TFC (11.85 mg/g dry weight (DW)) was recorded when cultures were elicited on day 10 [47]. A possible reprogramming of the phenylpropanoid pathway through this AgNPs elicitation was suggested since the accumulation of other phenylpropanoids such as lignans and neolignans was also affected [47].

Table 1. Role of silver NPs (AgNPs) as an elicitor of flavonoids.

Plant Species	Type of In Vitro Culture	Growth Media	NP Size (nm)	Effective Concentration of NPs	Effect on Flavonoid Production	References
<i>M. charantia</i>	Cell suspension culture	MS media	50	5 mg/L	Enhanced production of quercetin, kaempferol, catechin, myricetin, rutin, naringenin and biochanin A.	[36]
<i>C. anguria</i>	Hairy root	MS media	-	2.0 mg/L	Increase in flavonol production.	[39]
<i>B. rapa</i>	Hairy root	MS media	2–10	100 mg/L	Increase in TFC and accumulation of myricetin, naringenin, catechin, quercetin, rutin, kaempferol, biochanin A, formononetin, and hesperidin.	[40]
<i>A. caudatus</i>	Whole plant	Garden soil	17–91	50 ppm	Improved flavonoid accumulation, predominantly kaempferol and quercetin.	[41]
<i>C. tuberculata</i>	Callus	MS media with 2,4-D and BA	40	90 μ g/mL	1.8 mg TFC production.	[42]
<i>A. thaliana</i>	Seedlings	MS media	8–47	50 μ M	56% increase in anthocyanins.	[46]
<i>L. usitatissimum</i>	Cell suspension	MS media	18	-	TFC was 11.85 mg/g DW	[47]
<i>P. vulgaris</i>	Callus	MS media with NAA	Ag + Au	1:3 ratio	Enhanced TFC.	[48]
	Cell suspension	MS Media with NAA	Ag + Au	1:3 ratio	Enhanced TFC.	[49]

MS: Murashige and Skoog Basal Medium; BA: 6-Benzylaminopurine; NAA: 1-Naphthaleneacetic acid; TFC: total flavonoid content; DW: dry weight.

The addition of different NPs in combination is also effective in inducing stress in plants. To test this hypothesis, different ratios of AgNPs and gold NPs (AuNPs) alone or in combination with naphthalene acetic acid (NAA) were investigated for callus culture development and the production of phenolics and flavonoid compounds in *Prunella vulgaris*. As a consequence, the Ag-AuNPs (1:3) in combination with NAA induced maximum accumulation of flavonoid (6.71 mg/g-DW) content [48]. Likewise, enhanced flavonoids (7.62 mg/g-DW) were produced in *P. vulgaris* cell suspension culture [49].

3.2. CuNPs and CuONPs as a Novel Elicitor of Flavonoids

CuNPs possess unique physical resistance, electrical conductivity and chemical reactivity linked to their small size and high specific surface area [50]. The foliar spray of CuNPs in different concentrations (50, 125, 250, 500 mg·L⁻¹) on tomato showed an increase in flavonoid contents in tomato fruit. The highest content of flavonoids in the tomato fruits was obtained with the application of 50 mg·L⁻¹ of CuNPs, exceeding the control by 36.14%. This suggests that low doses of CuNPs generate metabolic effects due to the Cu accumulation and ROS generation [51]. *Ocimum basilicum* or basil is an important medic-

inal herb cultivated around the world which is enriched with bioactive compounds. A comparative study suggested the elevated regeneration capacity of basil plant via somatic embryogenesis using CuNPs as compared to copper sulfate solution [52]. Moreover, copper sulfate NPs (CuSO_4 NPs) enhanced the flavonoid content in *O. basilicum* seeds [53].

CuONPs are also reported to elicit in vitro cultures for enhanced flavonoid production (Table 2). Nano-CuO stress on Sriani Barley (*Hordeum vulgare*) leaves exhibited a significantly increased flavonol level (~1.2-fold over the control) on day 20 after 20 days; however, chlorophyll content decreased significantly [54]. CuONPs upregulated the transcription of genes associated with oxidative stress, therefore enhancing the phytochemical profile of *B. rapa* seedlings [55]. The cell suspension culture of *Gymnema sylvestre* when elicited with CuONPs (25–55 nm) resulted in a 2-fold increase in TPC and TFC [56]. Similarly, a higher TFC (9.450 and 12.878 mg QE g⁻¹ extract) in the shoots and roots of *Cichorium intybus* was observed upon nano-elicitation with CuONPs 20 days after treatment [57]. The highest flavonoid content, 23.076 ± 5.128 mg QE/g extract, was observed in the roots of CuONP elicited *Withania somnifera* plant 20 days after treatment [58].

The influence of CuONPs and polyvinyl pyrrolidone (PVP) and polyethylene glycol (PEG) impregnated CuONPs on seed germination, callus induction and the production of secondary metabolites in the shoots and roots of *Trigonella foenum-graecum* was studied in a comparative manner. It was found that PVP and PEG impregnated CuONPs were less toxic for calli induction. Furthermore, secondary metabolism was also enhanced as a result of nano-elicitation in a concentration dependent manner [59]. Despite the enhancement in bioactive compounds, a negative impact of nano-elicitation on biomass has also been reported. The influence of CuONPs on *A. thaliana* seedlings showed a significant reduction in plant biomass and total chlorophyll content, while anthocyanin content enhanced in response to all concentrations [60].

Stevia rebaudiana, called candy leaf, is a perennial herb which naturally protects from diabetes mellitus, hypertension and obesity [61]. Callus culture of *S. rebaudiana* was treated with CuONPs and ZnONPs in various concentrations. Phytochemical analysis revealed the highest TFC (2.23 µg/mg of DW) in response to 100 mg/L CuONPs, while the highest TFC (2.85 µg/mg of DW) was recorded for 100 mg/L ZnONPs. The lowest TFC was recorded in response to a 500 mg/L concentration of CuONPs as well as ZnONPs. Moreover, zero TFC was recorded when elicited with 1000 mg/L of both types of NPs. This trend suggests that stress increases by increasing the concentration of CuONPs or ZnONPs due to the formation of reactive oxygen species and enhanced secondary metabolism. However, after reaching a certain threshold, different physiological parameters of callus and the production of antioxidants begin to decline [62].

Bimetallic Cu-AuNPs were reported to enhance total flavonoid production (TFP-29.5 mg/L) in submerged root cultures of *S. rebaudiana* [63].

3.3. ZnONPs as a Novel Elicitor of Flavonoids

ZnONPs have also been investigated as abiotic elicitors of plant secondary metabolites (Table 3). Not only size, but also the morphology of NPs plays a role in effective elicitation. Licorice (*Glycyrrhiza glabra*) roots and rhizomes contain more than 100 various useful compounds, including phenolic compounds and glycyrrhizin [64]. In a study, the effects of bulk and nano sized ZnO and CuO on some secondary metabolites were compared in *G. glabra* seedlings. Generally, both bulk and nano-sized particles enhanced the secondary metabolism in comparison to the control. It was observed that nano-sized ZnONPs were efficient in eliciting the anthocyanin content compared to bulk sized ZnO and the control [65]. Due to their nanometer-size, NPs can easily penetrate plant cell walls and plasma membranes as well as the cytoplasm and cell organelles, therefore affecting the plant metabolism effectively [66].

Table 2. Copper NPs (CuNPs) and copper oxide NPs (CuONPs) as elicitors of flavonoids.

Plant Species	Type of In Vitro Culture	Growth Media	NP Type	NP Size (nm)	Effective Concentration of NPs	Effect on Flavonoid Production	References
<i>S. lycopersicum</i>	Plantlets	Greenhouse	Cu	50	50 mg/L	99.67 mg QE 100 g ⁻¹ of dry weight flavonoid production.	[51]
<i>O. basilicum</i>	Seeds	MS media	CuSO ₄	-	10 µM/L	Enhanced total flavonoid production.	[53]
<i>H. vulgare</i>	Seedlings	MS media	CuO	<50	0.5 mM, 1 mM and 1.5 mM	Enhanced flavonol production on day 20.	[54]
<i>G. sylvestre</i>	Cell suspension culture	MS media	CuO	25–55	3 mg/L	TFC enhanced 2-fold.	[56]
<i>C. intybus</i>	Shoots and roots	MS media	CuO	-	1 ppm	Highest 9.450 and 12.878 mg QE g ⁻¹ extract TFC level in shoots and roots, respectively.	[57]
<i>W. somnifera</i>	Shoots and roots	MS media	CuO	-	1 ppm	TFC production increased.	[58]
<i>T. foenumgraecum</i>	Callus culture	MS media	CuO, PVP and PEG impregnated	47	5 mg/L	Flavonoid production enhanced.	[59]
<i>A. thaliana</i>	Seedlings	MS media	CuO	-	10–100 mg/L	Enhanced anthocyanin content.	[60]
<i>S. rebaudiana</i>	Callus	MS media	CuOZnO	-	100 mg/L 100 mg/L	Highest 2.23 µg/mg of DW TFC Highest 2.85 µg/mg of DW TFC	[62]
<i>S. rebaudiana Bertoni</i>	Submerged root culture	MS media with NAA	Cu-Au bimetallic	-	-	TFC increased.	[63]

MS: Murashige and Skoog Basal Medium; NAA: 1-Naphthaleneacetic acid; TFC: total flavonoid content; PVP: polyvinyl pyrrolidone; PEG: polyethylene glycol; QE: quercetin equivalent; DW: dry weight.

Table 3. Zinc oxide NPs (ZnONPs) as an elicitor of flavonoids.

Plant Species	Type of In Vitro Culture	Growth Media	NP Size (nm)	Effective Concentration of NPs	Effect on Flavonoid Production	References
<i>G. glabra</i>	Seedlings	Hoagland's solution	-	1 µM 1 µM	Both CuO and ZnO enhanced the production of tannins, anthocyanins, phenolics, flavonoids and glycyrrhizin.	[65]
<i>E. purpurea</i>	Callus	MS media	-	75 mg/L	Flavonoid production enhanced.	[67]
<i>L. usitatissimum</i>	Cell suspension	MS media	35	1–500 µg/L	123.83 mg/L highest TFC recorded.	[68]
<i>Stevia rebaudiana</i>	Shoot	MS media	34	1.0 mg/L	Increase in TFC.	[69]
<i>L. ledebourii</i>	Shoot	MS media	-	20–100 mg/L	Anthocyanin, phenolics and flavonoid production increased.	[70]
<i>S. tuberosum</i>	Plant	Potting soil	-	300, 500 ppm	Increase in anthocyanin and flavonoid content.	[71]
<i>S. rebaudiana</i>	Plant	Nutrient solution	-	75 mg/L	87.8% flavonoid production.	[72]
<i>S. rebaudiana</i>	Root culture	MS media	ZnO 20–30 CuO 25–30	-	TFC level enhanced when treated with 2 mg/L ZnONPs and 20 mg/L CuONPs.	[73]

MS: Murashige and Skoog Basal Medium; TFC: total flavonoid content.

Seventy-five milligrams per liter of biogenic ZnONPs was reported to produce 3.7 mg/g DW flavonoids as compared to commercially available micro-sized ZnO, which produced 3.01 mg/g DW flavonoids in callus culture of *Echinacea purpurea* [67]. Biogenic ZnONPs effectively elicited in vitro flavonoid production in a cell suspension culture of Flax [68]. The shoot culture of *S. rebaudiana* and *Lilium ledebourii* showed increased secondary metabolism and TFC in response to engineered ZnONP elicitation [69,70].

ZnONPs enhanced anthocyanin and flavonoid contents in potato plants at an effective concentration of 300–500 ppm; however, an increase in enzymatic and non-enzymatic antioxidants led to oxidative stress and toxicity to potato plants [71]. There was 87.8% flavonoid production in *S. rebaudiana* plants when stressed with plant-synthesized ZnONPs [72]. The physiological and metabolic responses of candy leaf were elicited as a defense against applied ZnONPs and CuONPs [73].

3.4. Various Metallic Oxide NPs as an Elicitor of Flavonoids

There are various other metallic oxide NPs reported as elicitors of flavonoids (Table 4). The effects of thidiazuron (TDZ) growth regulator and magnesium oxide NPs (MgONPs) on radish seeds under lead stress were evaluated. There was a significant increase in plant growth, phenolic and flavonoid contents, free radical scavenging activity, and lead phytoaccumulation in response to combined TDZ and MgONP treatment. Moreover, there was a positive correlation of flavonoids with dry biomass and lead accumulation [69,74]. Nickel oxide NPs (NiONPs), CuO and ZnO significantly enhanced anthocyanins, TPC and TFC in in vitro grown eggplant seeds in a dose-dependent manner [75].

Dracocephalum kotschy is a valuable source of rosmarinic acid and methoxylated hydroxyflavones which shows antioxidative and antiproliferative potential against various cancer cells [76]. The application of Fe₃O₄NPs induced secondary metabolic gene expression as well as enhanced flavonoid content in a hairy root culture of *D. kotschy* [77]. Fe₃O₄NPs led to a superior TFC (4.65 mg g⁻¹ DW) in the hairy roots of chicory [78]. Iron NPs (FeNPs) showed growth promoting effects on in vitro grown *S. rebaudiana* at lower concentrations (45 µg/L). However, higher doses (90 and 135 µg/L) of FeNPs were found detrimental to growth characteristics and development. Moreover, a higher concentration of FeNPs (135 µg/L) induced stress, which produced a higher TFC [79]. Recently, magnetic Fe₂O₃NPs in the presence of a static magnetic field (SMF) resulted in the enhanced production of rosmarinic acid, naringin, apigenin, thymol, carvacrol, quercetin and rutin, TPC, TFC, anthocyanins, lignin and malondialdehyde in a cell suspension culture of *D. polychaetum* [80]. The use of magnetic fields has recently been proposed for plant growth and the production of plant secondary metabolites since they are less hazardous for the environment [81].

The role of cerium oxide NPs (CeO₂NPs) on food quality is not well known yet. Therefore, the impact of CeO₂NPs on the secondary metabolism of *R. sativus* seed was analyzed. The results were not as promising, as NPs increased the level of antioxidant compounds and showed no effect on TPC and TFC [82]. *Nigella arvensis* was treated with different concentrations of Al₂O₃NPs, NiONPs, and TiO₂NPs for 21 days. A significant increase in TFC and TPC was found. Moreover, the maximum content of quercetin was observed in the shoot and root parts under 50 mg/L NiONPs with a 2.2- and 1.8-fold increase compared to the control group. However, the kaempferol content was significantly decreased in all treatments, except for 1000 mg/L NiONP treatment in the root parts, which was 2.9-fold higher than the control group. These findings suggest the potential use of NPs as elicitors, considering their effective concentration [83]. TiO₂NPs have also been reported to enhance phenolic and flavonoid content in embryogenic callus culture of *Cicer arietinum* [84].

The stimulation of *D. kotschy* hairy roots by silicon dioxide NPs (SiO₂NPs) led to a significant increase in the induction and production of important pharmaceutical compounds including anticancer flavonoids, including cirsimaritin, xanthomicrol and isokaempferide [85]. SiO₂NPs also elicited in vitro hairy root cultures of *H. reticulatus* and *H. pusillus* [86]. Manganese oxide NPs (Mn₂O₃NPs) at a lower concentration promoted plantlet growth and enhanced the biosynthesis of the regulation of pharmacologically active metabolites by the activation of specific antioxidant enzymes. However, at higher concentrations (50–200 mg/L), Mn₂O₃NPs exhibited oxidative stress and toxicity in an in vitro plant culture of deadly nightshade [87].

Table 4. Various metallic oxide NPs as elicitors of flavonoids.

Plant Species	Type of In Vitro Culture	Growth Media	NP Type	NP Size (nm)	Effective Concentration of NPs	Effect on Flavonoid Production	References
<i>R. sativus</i>	Seed	MS media	MgO	-	20 mg/L	Increased plant biomass, TFC and antioxidant activity.	[74]
<i>S. melongena</i>	Seed	Filter paper	ZnO NiO CuO	18–20 10–20 25–55	100–1000 mg/L	Anthocyanin, TFC enhanced.	[75]
<i>D. kotschy</i>	Hairy root	MS media	Fe ₂ O ₃	-	75 mg/L	Enhanced flavonoid production.	[77]
<i>C. intybus</i>	Hairy root	MS media	Fe ₃ O ₄ ZnO	-	50 mg/L 50 mg/L	TFC increased in response to Fe ₃ O ₄ NPs only.	[78]
<i>S. rebaudiana</i>	Micro-propagation	MS media supplemented with sucrose (3%) and BA	Fe	<50	135 µg/L	TFC level was 1.6 mg/g DW.	[79]
<i>D. polychaetum</i>	Cell suspension	-	SMF Fe ₂ O ₃	-	100 ppm	Naringin, rosmarinic acid, carvacrol, apigenin, quercetin, thymol, rutin, TPC, TFC, lignin, anthocyanins and malondialdehyde production increased compared to control. Increase in antioxidant compounds and no effect on TFC.	[80]
<i>R. sativus</i>	Seed	Potting mix	CeO ₂	8	250 mg/kg	TFC, glucanase, quercetin and kaempferol content increased.	[82]
<i>N. arvensis</i>	Seed	Hoagland's solution	Al ₂ O ₃ , NiO, TiO ₂	5 5–8 20	0–2500 mg/L	Flavonoid and phenolic content increased.	[83]
<i>C. arietinum</i>	Embryonic callus	MS media	TiO ₂	-	6 mg/L	Rosmarinic acid, cirsimaritin, isokaempferide and xanthomicrol production increased 2-fold.	[84]
<i>D. kotschy</i>	Hairy root	MS Media	SiO ₂	100	-	Highest TFC 4.06 mg GAE/g FW on 24 h exposure.	[85]
<i>H. reticulatus</i> and <i>H. pusillus</i>	Hairy root	MS media	SiO ₂	10–100	50 mg/L	4.49-fold higher flavonoid production than negative control.	[86]
<i>Atropa belladonna</i>	Plant culture	MS media	Mn ₂ O ₃	30	100 mg/L		[87]

MS: Murashige and Skoog Basal Medium; BA: 6-Benzylaminopurine; TFC: total flavonoid content; DW: dry weight; FW: fresh weight; GAE: gallic acid equivalent.

3.5. Carbon-Based Nanomaterials as an Elicitor of Flavonoids

The most commonly employed carbon nanomaterials include single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), fullerenes and chitosan-NPs (Table 5). The discovery of MWCNTs, with their nearly perfect cylindrical structure of seamless graphite, together with the equally remarkable high aspect ratio SWCNTs, has led to an intense interest in these remarkable structures [88]. The in vitro callus culture of *Satureja khuzestanica* was elicited with different concentrations of MWCNTs (0–500 µg/mL). At lower concentrations (25 and 50 µg/mL) of MWCNTs, maximal callus growth and biomass accumulated, while at higher concentrations (100 and 250 µg/mL), the maximum level of secondary metabolites accumulated. Maximum flavonoids (21.48 mg CE/g DW) accumulated at 100 µg/mL MWCNT concentration [89]. MWCNTs enhanced the TPC, TFC, rosmarinic acid, antioxidants and PAL activity in seedlings of *S. verticillata* and *Thymus daenensis* [90,91]. A group of researchers reported that SWCNTs resulted in a higher production of flavonoids (20.66 mg QE/g DW) in plantlets of *Simmondsia chinensis* than the control [92].

Chitosan is a modified biopolymer, derived by the partial deacetylation of chitin, which acts as a defense elicitor [93]. Chitosan has been reported to activate pathogenesis genes and plant metabolism-related genes [94]. Chitosan-NPs increased the accumulation of phenolics and flavonoids by 20% and 24%, respectively, in shoot culture of *Camellia sinensis* [95].

Table 5. Carbon-based nanomaterials as elicitors of flavonoids.

Plant Species	Type of In Vitro Culture	Growth Media	NP Type	NP Size (nm)	Effective Concentration of NPs	Effect on Flavonoid Production	References
<i>S. khuzestanica</i>	Callus	B5 basal	MWCNTs	-	100 µg/mL	2.6-fold higher TFC than control.	[89]
<i>T. daenensis</i>	Seedlings	MS media	MWCNTs	50 µm	0–2000 µg/mL	Increased TFC, antioxidant and PAL activity	[90]
<i>Salvia verticillata</i>	Seedlings	Potting mix	MWCNTs	-	0–1000 mg/L	Secondary metabolism enhanced.	[91]
<i>S. chinensis</i>	Plantlets	MS media	SWCNTs	110–170	0.002 g/L	Flavonoid contents were increased.	[92]
<i>Camellia sinensis</i>	Foliar spray on leaves	Hydroponic	Chitosan-NPs	40–180	0.01	Increase in phenolic and flavonoid accumulation.	[95]

MWCNTs: multi-walled carbon nanotubes; SWCNTs: single-walled carbon nanotubes (SWCNTs), MS: Murashige and Skoog basal medium; B5: Gamborg B5 basal medium; TFC: total flavonoid content; PAL: L-phenylalanine ammonialyse.

4. Conclusions and Future Perspectives

Elicitation is a promising strategy to over-produce industrially important flavonoids. Amongst different types of elicitors, NPs as elicitors are effective in lower concentrations to enhance the productivity of flavonoids. Despite immense research on NPs as novel elicitors of flavonoids, there are still some knowledge gaps which need to be filled. Firstly, a deep understanding of the underlying mechanism of nano-elicitation is important for its successful industrial adoption. Moreover, an understanding of plant response to the nanomaterials applied as elicitors either individually or in combination with other classes of NPs or with other types of elicitors is essential for developing strategies for enhanced flavonoid production [31].

Negative effects on plant primary metabolism such as lower chlorophyll content, a decrease in cell viability, a decrease in sugar concentration and suppressed seed germination have been reported during nano-elicitation experiments [25]. To ensure the success of elicitation, addressing nano-toxicity in plants is important. Furthermore, the targeted delivery of NPs enhances the efficacy of elicitation using lower concentration of NPs and prevents phyto-toxicity. This can be achieved by the surface functionalization of NPs with bio-molecules, tags or magnetic fields [96].

Moreover, in most of the studies, an enhancement in the production of flavonoids is reported only in terms of total flavonoid content. This gives an overall picture of NPs' role as an elicitor of flavonoids. However, from an industrial viewpoint it is important to characterize and report the effect of NPs on specific industrially important flavonoids. There are some plant species naturally enriched with flavonoids, such as *Allium* species, which have not been treated with NPs to elicit flavonoid production [97]. Further analysis of different plant species will allow the discovery of novel flavonoids which can be nano-elicited in in vitro cultures for the enhanced production of flavonoids for industrial use.

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