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PREFACE

Application of nanoparticles in plant tissue cultures: minuscule size but huge effects

S. Ochatt[1] · M. R. Abdollahi[2] · M. Akin[3] · J. J. Bello Bello[4] · K. Eimert[5] · M. Faisal[6] · D. T. Nhut[7]

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Reliable and efficient strategies for plant regeneration are the prerequisites for reproducible and successful propagation, conservation, gene transfer and enhanced secondary metabolite production in vitro. In this respect, treatments with nanoparticles (NPs) studied in recent years have successfully eliminated microbial contaminants from explants, with a parallel positive impact on callus proliferation, but also on the induction of organogenesis, somatic embryogenesis, somaclonal variation, in vitro conservation, genetic transformation, and secondary metabolite production.

This Special Issue (SI) of PCTOC focuses on this emerging in vitro technology, as well as on the study of the potential hormetic response, toxicity concerns and safety issues resulting from the use of NPs in plant tissue cultures. It includes three comprehensive review papers and sixteen original articles.

In an authoritative review, **Inam et al.** surveyed the literature concerning the use of metal oxide NPs as nanoelicitors for secondary metabolite production. Recent years have seen an increasing interest in the production and uses of metal oxide nanoparticles for various purposes, among which are the improvement of the production of secondary metabolites by cultured cells and callus of a range of species. Secondary metabolites accumulate in tissues as a defense reaction viz. a viz. of several abiotic stress agents, including salinity, drought, and extreme temperatures among others. In this review, the authors examined the different routes of exposure of metal oxide NPs in plants, and also their role as novel elicitors of important phenols, flavonoids, alkaloids, and terpenes, with relevant metabolic functions. Interestingly, they critically discussed the mechanism underlying nano-elicitation and NP uptake and translocation in plants, proposing future research directions.

A comprehensive review by **Sena et al.** discussed the applications of green synthesized NPs in medicinal plant research. This eco-friendly approach to produce NPs is a viable, quick, and effective strategy. The use of NPs has sometimes been suggested to encompass a certain level of toxicity due to the methods used for their obtention and green synthesis appears as the logical alternative to contour this but, rather surprisingly, though, it has only seldom been researched. The authors also delved into the significance and uses of NPs within the context of secondary metabolites production, as well as their notable antioxidant, antibacterial, and antimicrobial activities, which can also accelerate plant development, enhance photosynthetic efficiency, and

✉ S. Ochatt
sergio.ochatt@inrae.fr

M. R. Abdollahi
m.abdollahi@basu.ac.ir

M. Akin
akinmeleksen@gmail.com

J. J. Bello Bello
jericobello@gmail.com

K. Eimert
Klaus.Eimert@hs-gm.de

M. Faisal
faisalm15@yahoo.com

D. T. Nhut
duongtannhut@gmail.com

[1] Agroécologie, InstitutAgro Dijon, INRAE, Univ. Bourgogne, Univ. Bourgogne Franche-Comté, 21000 Dijon, France

[2] Bu-Ali Sina University, Hamedan, Iran

[3] Iğdõr University, Iğdõ, Turkey

[4] CONAHCYT, Colegio de Postgraduados Campus Córdoba, Veracruz, Mexico

[5] Hochschule Geisenheim University, Geisenheim, Germany

[6] King Saud University, Riyadh, Saudi Arabia

[7] Vietnam Academy of Science and Technology, Dalat, Vietnam

improve the plant performance in general. They highlighted a possible hormetic effect or hormesis of the studied NPs on plant development, that can be defined as "a stimulatory process of low dose and inhibition at high doses of NPs". It has been stated that low concentrations of NPs induce hormetic effects through activating plant stress defence mechanisms. This paper discusses how NPs act depending on the precise particle size, composition, concentration, and application method, areas that still require more research input for a better comprehension of the mechanisms underlying their action.

Humbal and Pathak summarized the state-of-the-art of the application of various metallic, bimetallic, non-metallic, carbon-based, and composite NPs as elicitors of economically important medicinal secondary metabolites in different species. They briefly explained the exposure, uptake, and translocation of nanoparticles inside the plant cell and discussed the possible mechanisms of nanoparticle-mediated elicitation of secondary metabolites in plant tissue cultures.

Of the various NPs used in plant tissue cultures, metal NPs have been more frequently studied, and among them silver NPs (AgNPs) are the most common in the literature. It is hence not surprising that five articles in this SI concerned the use of AgNPs with different species and for different purposes.

Truong et al. reported the enhancement of plant regeneration competence from leaf and internode explants through thin cell layer culture in purple passion fruit (*Passiflora edulis* Sims f. *edulis*) using AgNPs. They found that 1.5 mg L^{-1} AgNPs associated with BAP favoured the largest regeneration responses from the leaf explants, while for internode explants there was a notable topophysis effect, whereby the position of the internode within the stem affected the regeneration competence of the explants thereof, likely correlated with the endogenous hormone concentration at different node positions. Moreover, it was found that the addition of 3.0 mg L^{-1} AgNPs significantly enhanced the proliferation and maturation of somatic embryos from thin cell layer internode explants.

In another study from the same team, **Cuong et al.** showed that AgNPs significantly improved the micropropagation of *Limonium sinuatum* (L.) Mill. 'White', both for explant surface disinfection, but also for the in vitro growth, development and acclimatization of produced plants. Noteworthy, a modulating effect of AgNPs was recorded on endogenous hormone content during the shoot multiplication and rooting stages of plantlets. Explants treated with 200 mg L^{-1} AgNPs for 20 min exhibited a better disinfection and shoot induction than those disinfected with 1000 mg L^{-1} $HgCl_2$ for 5 min (of relevance in the wake of potential limitations for the use of $HgCl_2$ in several countries). The explants cultured in presence of 1.0 mg L^{-1} AgNPs produced more shoots/explant than the control by reducing ethylene content and increasing zeatin content. Likewise, a medium with 0.4 mg L^{-1} AgNPs shortened the delay for rooting of plantlets indicating a low ethylene content and high content of IAA, GA_3, and ABA compared to the untreated controls, and the resulting plants also showed better greenhouse acclimatization than those of the control.

On the other hand, **Manokari et al.** reported that AgNPs improved the in vitro propagation responses of *Gaillardia pulchella* cv. 'Torch Yellow', with optimum shoot proliferation and shoot biomass for explants cultured on MS medium supplemented with 0.5 mg L^{-1} BAP, 0.25 mg L^{-1} IAA and 4.0 mg L^{-1} biogenic AgNPs as compared with the control. In addition, shoots developed on AgNPs-containing medium were healthy, sturdy, and greener than the controls produced on media lacking AgNPs, that were hyperhydric and chlorotic. AgNPs enhanced chlorophylls and carbohydrate contents and reduced carotenoids in the leaves, also improving root induction and the acclimatization of in vitro propagated plantlets. This likely occurred through an improved organization of stomatal complexes and trichomes development which, in turn, enhanced the defence mechanism towards abiotic stress thereby helping the plantlets to survive during acclimatization and post-acclimatization under greenhouse and, later, field conditions.

Andújar et al. showed that AgNPs promoted dipertene production in *Stevia rebaudiana* cultures in temporary immersion bioreactors for 21 days. They showed that 25 and 37.5 mg L^{-1} AgNPs decreased shoot multiplication rate, shoot length, the number of nodes and leaves per shoot, and their fresh and dry weights compared with the control, while no negative effect was observed at a lower (12.5 mg L^{-1}) concentration. On the other hand, chlorophyll a, carotenoids and soluble phenolics were increased in plants supplied with 25 mg L^{-1} AgNPs, suggesting oxidative stress. The endogenous levels of diterpenes were significantly increased by the application of 12.5 mg L^{-1} AgNPs. Altogether, the results indicate the potential role of AgNPs as elicitors to promote diterpenes production in stevia, provided a balance is ensured between oxidative damage and secondary metabolite production.

Working with five different in vitro grown crops, **Tomaszewska-Sowa et al.** examined the cyto- and genotoxic side effects of using AgNPs as antimicrobial agents instead of standard sterilization methods. They tested the effects of a range of 50 to 100 mg L^{-1} AgNPs on endoreduplication, DNA content and growth of seedlings grown in vitro of rapeseed, white mustard, sugar beet, red clover, and alfalfa. The genome size and DNA synthesis patterns in the roots, hypocotyls, and leaves from seedlings of these species were established by flow cytometry. It was found that while AgNP-treatment did not influence germination or

genome size, it did increase root length and endoreduplication intensity, which could be interpreted as a response defence mechanism against stress provoked by the disruption of mitotic division by AgNPs.

Yoshihara et al. studied the effects of overexposure to metal oxide NPs on the root elongation and chlorophyll production in lettuce. In this appealing work, the authors exposed lettuce seedlings to Zn applied as ZnNPs and Zn^{2+} ion in aqueous solutions. Thus, 0.74 mg L^{-1} Zn^{2+} ions provided as 10 mg L^{-1} ZnNPs inhibited root elongation while no such inhibition occurred when the same amount of Zn^{2+} ions dissociated from $ZnCl_2$ was provided. Dispersions of water insoluble SiO_2 and TiO_2 NPs did not affect root elongation, which suggests that the phytotoxicity effect observed was due to the ionizable metal oxide ZnONP dispersions. Indeed, the Zn content in lettuce roots incubated in ZnONP dispersions was much higher than for $ZnCl_2$ solution-incubated roots. A 20 mg L^{-1} ZnONPs dispersion reduced the chlorophyll contents of seedlings, and all plants died after transplanting onto a ZnONPs-free medium. Inhibition of root elongation was accompanied by an accumulation of water-soluble components of the cell walls in roots through a specific mechanism.

Also working with ZnONPs, **Canales-Mendoza et al.** evaluated the in vitro multiplication responses of *Agave salmiana* var. Ayoteco. They used ZnONPs of an average size of 70 nm biosynthesized from cell-free filtrate from *Mucor fragilis*. A 20-day treatment with ZnONPs promoted organogenesis and modified the structures in shoots and seedlings, mainly the stomata. This occurred without the accumulation of ZnO and was coupled with antioxidant activity in such tissues that depended on the stress generated by abiotic agents and on the NPs to which they were exposed.

In two independent articles, **Hanif et al.** reported the use of NPs to improve the drought tolerance induced by 5% and 10% PEG stress in *Coriandrum sativum*. In the first study, proline coated ZnONPs were assessed as a nanofertilizer against drought stress. The authors characterized by scanning electron microscopy (SEM) and powder X-ray diffraction (XRD) ZnONPs with hexagonal structures of 14.73 and 20.59 nm, which significantly increased the shoot and root length as well as the dry weight of plants grown under stress. Moreover, the biochemical and antioxidant profile of such plants demonstrated the stress alleviating effect of the ZnOPNPs, through a decreased contents of phenolics and flavonoids as NPs concentration increased. At 100 mg L^{-1}, ZnOPNPs reduced the free radical scavenging activity in shoots and in root, while the total antioxidant profile decreased due to the improvement in antioxidant enzyme activity which reduced drought stress in the coriander plants. In a follow-up article, **Hanif et al.** studied the synergistic effect of a glycine betain-ZnO nanocomposite in coriander. Thus, ZnONPs were coated with glycine betaine (ZnOBtNPs), SEM and XRD showed that the ZnONPs were slightly smaller and spherical compared with the ZnOBtNPs which were larger and hexagonal, while Fourier transform infrared spectroscopy (FTIR) confirmed ZnO-Betaine formation. ZnOBtNPs at 100 mg L^{-1} significantly increased the shoot and root length as well as the fresh weight of drought-stressed plants, whereas a higher concentration of ZnOBtNPs determined a stress mitigating response, as shown by a decreased phenolic and flavonoid contents and a reduced oxidative damage coupled with the up-regulation of the antioxidant defence systems. The authors also observed a decrease in free radical scavenging activity and reducing potential in plantlets following NPs application.

The use of NPs as decontaminants is frequent in medicine but has been scarcely applied to plant tissue cultures. **Rakhimol et al.** formulated casein stabilized and AgNPs, gold (AuNPs) and copper oxide (CuONPs) NPs as decontaminants to eliminate endophytic bacteria from in vitro cultures of *Scoparia dulcis*, where it is a major constraint. The synthesized AgNPs and AuNPs were spherical shape with an average diameter of 13.5 nm and 3.5 nm, respectively, while CuONPs were spindle shaped with an average thickness of 25 nm. First, the authors isolated and identified the bacterial endophytes of *Scoparia dulcis* through 16 S rRNA sequencing. Then, dose-response analyses revealed that the Minimum Inhibitory Concentration of AgNPs, AuNPs and CuONPs against all endophytic bacterial contaminants was, respectively, of 0.125, 0.25 and 0.25 mg mL^{-1} while the Minimum Bactericidal Concentration for all was 1 mg mL^{-1}. Hence, all three AgNPs, AuNPs and CuONPs proved to be effective and lethal against all the isolated bacterial endophytes.

In their work with the quince rootstock QA, **Farhadi et al.** compared rice husk-derived biogenic silica NPs (SiO_2NPs) and ZnONPs as additives to improve the growth and proliferation of shoot cultures during a 35-day period. They found that in vitro shoots treated with 1 mg L^{-1} SiO_2NPs had the highest number of axillary shoots, while plantlets regenerated from media with 2.5 mg L^{-1} ZnONPs exhibited the highest shoot length and number of leaves.

Sharma et al. assessed the use of SiO_2NPs as elicitors to increase the production of rebaudioside-A (reb-A) by plants of *Stevia rebaudiana* micropropagated on solid and liquid cultures. Although the authors could not find any clear uniform pattern for all the parameters examined with the different treatments tested, they could observe that various morphological traits (shoot number, shoot length, node number, leaf area and fresh weight) were higher in liquid than in solid cultures and, this, irrespective of the SiO_2NPs treatment applied. Conversely, solid cultures had a higher chlorophyll and carotenoid content than liquid cultures, the

same as for the antioxidant activity, indicative of higher stress for shoots cultured on solid medium where plants also exhibited a significantly higher content of reb-A than those in liquid medium. Moreover, in the solid medium the reb-A content increased further in presence of SiO_2NPs, while the reverse occurred in liquid medium. These results suggest that the mechanism of uptake and action of SiO_2NPs in solid and liquid medium is likely to differ.

Using cobalt NPs (CoNPs) in the medium, **Van The Vinh et al.** assessed the stem elongation and competence for somatic embryogenesis as also the subsequent in vivo growth and flowering of tuberous begonias (*Begonia x tuberhybrida* Voss) grown under different light sources (fluorescent lamps - FL, blue LED, red LED, and blue to red LED ratio). After 60 days of culture, shoots cultured under red LEDs were longer, had more internodes per shoot, and larger fresh and dry weights compared to those kept under the other light sources. On the other hand, cultures under red LED exhibited higher somatic embryogenesis, with a larger number of somatic embryos, and a higher percentage of with torpedo-shaped and cotyledonary somatic embryos compared to those under other light sources. Culture of such cotyledonary somatic embryos on a medium containing 0.0465 µg L^{-1} CoNPs enhanced plantlet growth, acclimatization, and flowering of plantlets in the greenhouse.

Adabavazeh et al. studied the elicitation of secondary metabolites production from hairy roots of *Calotropis procera* by supplementing cultures with various concentrations of synthesized Fe_3O_4NPs and salicylic acid (SA) to improve their growth and productivity. The addition of Fe_3O_4NPs to leaf explant-derived hairy roots determined an increase of growth, soluble sugars, total proteins, and antioxidant enzymes and reduced H_2O_2 and MDA levels. This effect was significantly greater for hairy roots treated with both Fe_3O_4 NPs and SA together, than in those exposed to the elicitors individually. Such transformed hairy roots of *C. procera* had a significantly larger production of essential oil than the intact plant, especially when supplemented with Fe_3O_4 NPs and SA.

In a separate study of the potential of NPs to improve the production of secondary metabolites of medicinal interest, **Ambreen et al.** examined the effects of Carbon nanotubes (CNTs) on adventitious roots of *Nigella sativa*. They revealed that the application of CNTs at 5.0 to 20.0 mg L^{-1} significantly enhanced the number of roots induced and their fresh biomass on solid medium. Subsequent experiments using shaken liquid cultures showed that a 4-hour pre-treatment with 10.0 mg L^{-1} CNTs permitted the highest root proliferation. Similarly, 2-hour and 4-hour pretreatments resulted in a higher total phenolic and flavonoid content in the adventitious roots than an 8-hour pre-treatment, with optimum results for the 4-hour pretreatment with 25.0 mg L^{-1} CNTs. In addition, the DPPH antioxidant activity increased while Phenyl alanine ammonia lyase (PAL) activity decreased with higher CNT concentrations and longer pretreatment durations. In any case, the adventitious roots of *N. sativa* treated with 5.0 mg L^{-1} CNTs exhibited elevated levels of α-thujene, β-pinene, d-limonene, p-cymene, α-terpineol, carvone, and β-Elemene, coupled with significant levels of thymoquinone, thymol (6.4%), and carvacrol (2.3%).

Finally, **Allah et al.** examined the effects of Chitosan NPs on the growth and genetic transformation of *Phoenix dactylifera*. Cultures of three commercial date palm varieties were transformed with the *AT1G12660* "*Thio-60*" gene to introduce resistance to fungus infection. Chitosan NPs were efficient in favouring the genetic transformation in all three varieties, as verified by PCR, and the subsequent inoculation of the transgenic plants produced with *Fusarium oxysporum* showed that they had become resistant after their transformation with the *Thio-60* thionin gene.

The constant growth of population added to climate change have led the UN to propose a series of Sustainable Development Goals which will require the development and exploitation of novel approaches and techniques for crop production, but also the optimisation of existing ones. In this context, the advent of nanotechnology provides a range of novel strategies to improve not only seed germination, but also plant growth, associated with a better tolerance to stress and the potential to improve the production of secondary metabolites of medicinal interest. It is precisely in this latter area that NPs have been most frequently studied, as elicitors to produce secondary metabolites. Rather surprisingly, though, this emerging area of research has not yet reached its climax and, currently, its application in food and agriculture remain scarce. Furthermore, much research input is still required about the beneficial and adverse effects of NPs and the evaluation of their hormetic effect on plant development (growth + differentiation) before this technology may be widely applied for several biotechnological applications and also become an innovative option for sustainable agriculture, used as nanofertilizers, nanopesticides, nanosensors, and agri-food agents.

This SI addressed most of these aspects of nanotechnology applied to in vitro plant tissue cultures and, as such, should be of appeal to the large readership of PCTOC.