CARBON-IRON MAGNETIC NANOPARTICLES FOR AGRONOMIC USE IN PLANTS: PROMISING BUT STILL A LONG WAY TO GO

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In the recent years, multiple ways of interaction between the fields of nanotechnology and biology have been opened, mainly in the biomedical research, with the development of tools for diagnosis and controlled delivery of substances (1, 2). On the other hand, in the field of plant biology, the interaction between both disciplines has been less frequent. Most of the published work on this field has focus in the environmental impact of nanoparticles on crop growth and development (3, 4); and also on the bio production of nanoparticles using plant extracts (reviewed in 5, as an example see also 6, 7, 8). Much less attention has taken other possible aspects of the interrelationship between nanotechnology and plant biology, such as the development of nanodevices for controlled delivery of drugs or different kind of substances (9, 10), in a similar way to that already developed in the medical research.

Recently, our group has developed an approach for the application of carbon-coated iron nanoparticles to pumpkin plants. The goal of this project was the development of tools for the treatment of pathologies affecting specific areas or organs of the plants. To achieve that, nanoparticles carrying the phyto-remedy will be applied to the affected plants, and magnetic fields will be applied to the organs of the plant affected by the pathology. In this way, the nanoparticles will be retained in the affected area, and the effect of the active compound linked to the nanoparticle will be concentrated in a restricted area.

In a previous report, we described the capability of different microscopic methodologies to identify and locate nanoparticles in plant tissue samples, including both electron microscopy and light microscopy approaches (11). Using this knowledge, we used a correlative microscopy approach, identifying first the presence of nanoparticles in sections of resin-embedded tissue by light microscopy (bright field, phase contrast and dark field), followed by a further analysis of consecutive sections from the same block, this time by transmission

electron microscopy. This approach allowed us to unveil many different aspects of the behaviour of the nanoparticles in the living tissue. First of all, movement of the nanoparticles was detected at different levels: chains of nanoparticle-aggregates carrying cells were apparent close to the application point, when such application was made by 'injection' of the nanoparticle suspension into the pith cavity of the stem, suggesting the flux of nanoparticles from one cell to another. Also, after the same kind of application, nanoparticles were detected in an area close to the vascular core, but appearing as isolated particles in the cytoplasm of the cell. Furthermore, after application of the nanoparticle by 'spray' (that is, application of a drop of the solution over the surface of the leaf, close to the petiole insertion point), isolated nanoparticles were also detected close to the application point (Figure 1). This last data is particularly relevant, as this application method attempted to emulate that of breeders and coordinators of phytosanitary control. The fact that the nanoparticles are capable of penetrating through the leaf cuticule and into the cell cytoplasm opens the possibility for the use of this approach in phytosanitary applications.

A second question of special interest in our analysis was the differential response to the presence of the nanoparticles shown by the cell cytoplasm when they appear in the form of aggregates when compared with cells carrying non clustered nanoparticles. In fact, a dense cytoplasm with starch-containing organelles was observed concomitantly with nanoparticle aggregates in the cytosol (Figure 2), suggesting that plant cells could respond to the presence of a high density of nanoparticles by changing their subcellular organization. On the other hand, no response was observed in those cells in which only isolated nanoparticles were detected (Figure 1). The change on the cytoplasm of the cells was accompanied by the fact that the cell-to-cell movement of the particles in regions with a high density of aggregates

seems to direct them to the exterior of the organism, what points to a physiological response from the plant to the intracellular presence of nanoparticle aggregates.

Despite the obvious advance that supposes the possibility of the application of nanoparticles on agronomical applications, it is also clear that there are many aspects in the protocols for detection of the nanoparticles and for their infiltration into the plant than can be improved. As shown in our work, the detection of isolated nanoparticles is almost impossible to achieve just by the resolution of the conventional optical microscopy, and their detection by direct scanning of ultra-thin sections by electron microscopy is a tedious and time consuming task, especially if there is no clear evidence of the presence or not of nanoparticles in a certain part of the plant. Therefore, it is convenient to develop protocols with auxiliary methodologies to increase the sensibility of the microscopy techniques, that could allow directing the analysis by TEM to samples in which presence of nanoparticles has been previously assessed with certainty. Several methodologies have been employed for the detection of metallic nanoparticles in large tissue samples or in living tissues, with a special development in the field of therapy and diagnosis of diseases in the central nervous system. In this area magnetic resonance imaging (12, 13), phototermal interference contrast (14) and conventional iron staining (13) approaches have been successfully applied, although the resolution level is still low, with ranges between 1 μm and the cell size. A suitable solution could be the optimization of current methods for iron staining (Reviewed in 15), provided that lesser equipment requirements are needed than for the other approaches. In this line of action, a protocol should be optimized for their use to detect our particles in plant cells using as a starting point previous reports of iron detection in plant tissues, as the one used by Green (16) for detection of inorganic ferric iron in *Arabidopsis thaliana.* Also, as a suitable system for the detection of global uptake of magnetic nanoparticles into plant organs, a vibrating sample

magnetometer has been used successfully to measure the amount of nanoparticles taken by different organs of *Cucurbita maxima* plants (17), what could be a good option for the selection of samples of interest to be analyzed with microscopy in future experiments. Last, but not least, an even more straight forward approach in the attachment of coloured, fluorescent, or chemically detectable compounds to the nanoparticles, allowing an enhancement of the capacity of optical microscopy to detect those (18).

Our approach has been shown to allow the internalization of nanoparticles into plant cells in vivo, but the system as established initially is far from being functional, and several questions require improvement. First, the distribution of nanoparticles is still very limited, and most of the intracellular translocation of nanoparticles takes place near the application point, and much more efficiently when it is effectuated by injection. The most interesting way of application, the pulverization, has given only very limited results, with presence of intracellular nanoparticles but just in the first cellular layer, the epidermis. Also long range transport has still a low efficiency. Observations in samples equivalent to the ones that we have analysed of fresh vibratome section, in which nanoparticles seemed to be present (11) may point to the possibility of loosing some NPs during the processing for EM analysis. Again, this possibility reinforces the need of a pre-scan of the samples to identify the presence of nanoparticles before a further analysis.

Interestingly, it has been described recently the uptake of magnetite nanoparticles through the root system in *Cucurbita maxima* plants, as well as when applied by spraying, but the first method was tested only in plants growing in liquid media, and in the second only plants growing again in liquid media showed a significant uptake of particles (17). Also, in

Arabidopsis and *Phalaenopsis* plants, it has been possible to perform live imaging of the uptake of other kind of nanoparticles (NaYF4:Yb,Er) (19).

As stated previously, these experiments support the applicability of magnetic nanoparticles for use in agronomic purposes. But despite of the promising results, there is still a long way to go until they are suited for their use. This commentary has attempted to focus in some of the aspects to improve in the methodology employed.

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Figure Legends

Figure 1. Isolated nanoparticles localized close to the epidermis after spray application. a) Low amplification image of the area were the nanoparticle is (squared area): SEC sub estomatal cavity; GC guard cell; E exterior; LP lagunar parenchyma; EP epidermis. b) Detail of the region squared in a), showing an isolated nanoparticle (arrow). Bars in a: 5 μm, b: 200 nm.

Figure 2. Different behaviour between cell cytoplasm depending of the content in nanoparticle aggregates. a) low amplification image showing unmodified cells (UMC) without nanoparticles, next to a nanoparticle carrying cell (NCC), whose cytoplasm is full of organeles. b) Detail of the area squared in a), indicating the presence of nanoparticle aggregates (asterisks). c) Detail of a nanoparticle aggregate. Bars in a: 10 μm, b: 2 μm, c: 200 nm.

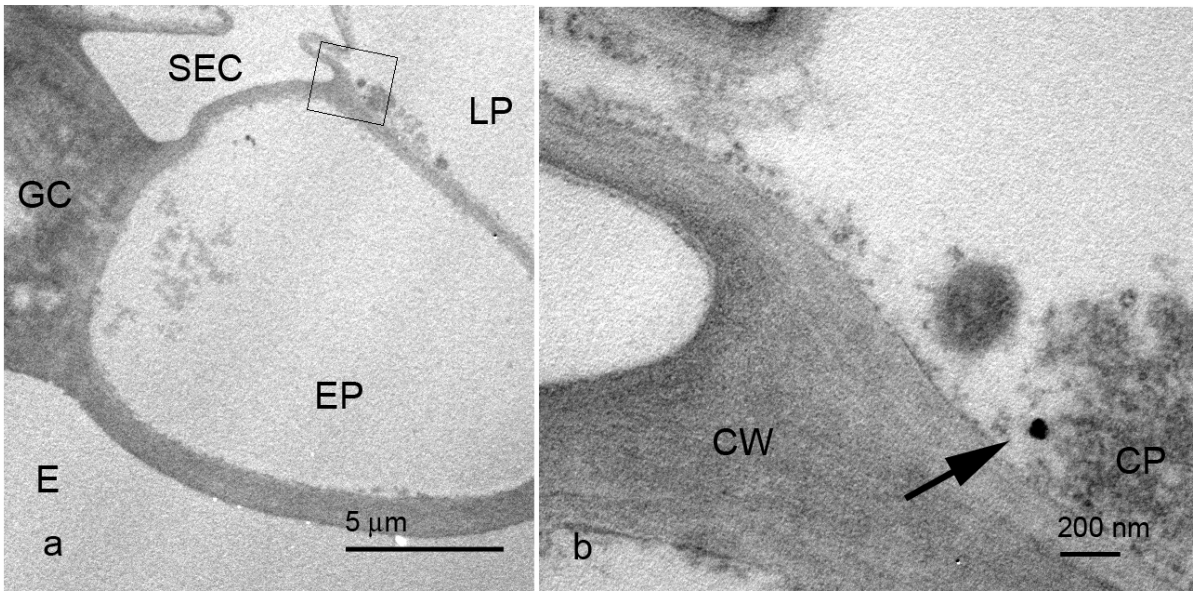

Figure 1

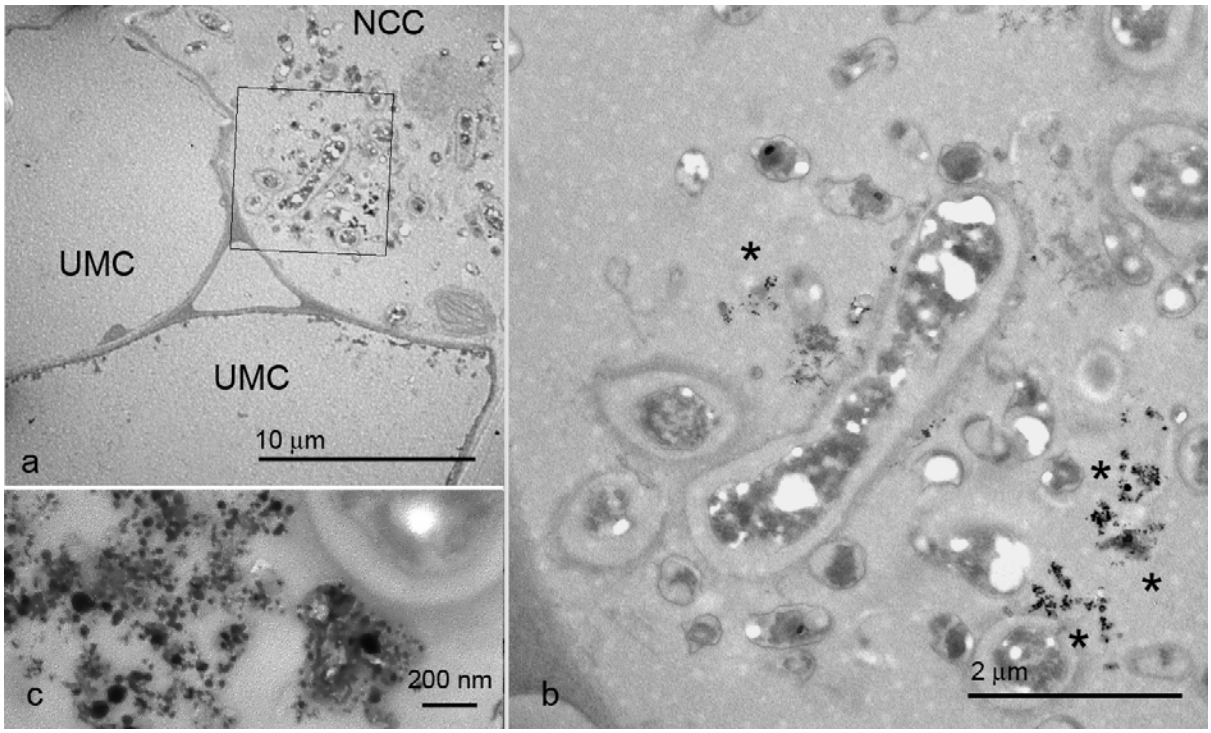

Figure 2