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ORIGINAL ARTICLE

First evidence on phloem transport of nanoscale calcium oxide in groundnut using solution culture technique

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Abstract Nanoscale materials, whose size typically falls below 100 nm, exhibit novel chemical, physical and biological properties which are different from their bulk counterparts. In the present investigation, we demonstrated that nanoscale calcium oxide particles (n-CaO) could transport through phloem tissue of groundnut unlike the corresponding bulk materials. n-CaO particles are prepared using sol–gel method. The size of the as prepared n-CaO measured (69.9 nm) using transmission electron microscopic technique (TEM). Results of the hydroponics experiment using solution culture technique revealed that foliar application of n-CaO at different concentrations (10, 50, 100, 500, 1,000 ppm) on groundnut plants confirmed the entry of calcium into leaves and stems through phloem compared to bulk source of calcium sprayed (CaO and $CaNO_3$). After spraying of n-CaO, calcium content in roots, shoots and leaves significantly increased. Based on visual scoring of calcium deficiency correction and calcium content in plant parts, we may establish the fact that nanoscale calcium oxide particles (size 69.9 nm) could move through phloem tissue in groundnut. This is the first report on phloem transport of nanoscale calcium oxide particles in plants and this result points to the use of nanoscale calcium oxide particles as calcium source to the plants through foliar application, agricultural crops in particular, as bulk calcium application through foliar nutrition is restricted due to its non-mobility in phloem.

Keywords Nanotechnology · Nanomaterials · Calcium oxide · Phloem · Mobility · Groundnut

Introduction

Nanoparticles are atomic or molecular aggregates posing modified physical–chemical properties compared to the bulk materials and having less than 100 nm measured size in at least one dimension (Nel et al. 2006). In the recent decade, nanomaterials have received tremendous attention for their positive impact in improving many sectors including consumer products, pharmaceutics, cosmetics, energy, medicine, etc. (Roco 2003). But limited reports are available on the positive effects of nanomaterials on plants (Prasad et al. 2012), agricultural plants in particular. Pronounced effect on increase in root and shoot length as well as accumulation of biomass in green gram was recorded for nanomaterial-treated plant as compared to control. Maximum effect was found at 50 ppm ZnFeCu-oxide followed by 50 ppm FeO and least for 20 ppm ZnO (Dhoke et al. 2013). Studies on the toxicity of nanomaterials are still emerging and basically evidence several negative effects on growth and development of plants (Monica et al. 2011). Several reports confirmed the potential toxic effects of nanomaterials on higher plants (Lu et al. 2002; Lin and Xing 2007; Lee et al. 2008; Ma et al. 2010).

The concept that nanomaterials could be of useful in agricultural systems is a relatively new one and under

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development now. Nanotechnology in agriculture would be of interest of developing countries, promising to reduce hunger, malnutrition and child mortality. The application of nanomaterials in agriculture aims to reduce nutrient losses in fertilization and to increase yields through nutrient management (Srilatha 2011: Sharon et al. 2010: Garica et al. 2010; Rashidi and Khosravi-Darani 2011). Several factors that influence the efficiency of fertilizers could be addressed using nanomaterials. Nanomaterials applications in agriculture and their potential advantages are evident through reports in the literature (Srilatha 2011; Ghormade et al. 2011; Garica et al. 2010). For example, nanomaterials with high surface area and with appropriate sorption properties may minimize losses by reducing runoff and with slow releasing kinetics. Specially designed nanoparticles could enhance the uptake and translocation of nutrients in the plants.

Use of nanomaterials in agriculture—specifically in plant nutrition—may pose unforeseen risks because these applications have the potential to release nanomaterials into the environment. Human and environmental exposure to nanomaterial residues in crops and soil increases accordingly due to the bioaccumulation of nanomaterials in the environment and food chain. As agriculture is aiming to sustainable management of natural resources, the application of nanomaterials must be critically evaluated towards safety of their usage in agriculture. Most importantly, their application methods must be standardized for low input fluxes and to minimize the environmental hazards thereby.

Calcium is an essential plant nutrient that plays structural role in the cell wall and membranes and regulates plant growth and development (Helper 2005). It acts as a critical part of the cell wall and forms strong structural rigidity by forming cross-links within the pectin polysaccharide matrix. Along with the plant growth, the structural integrity of stems that hold flowers and fruit, as well as the quality of the fruit produced, are strongly coupled to calcium availability. Further, calcium enhances the disease resistance in plants against bacterial and viral diseases (Usten et al. 2006).

The most critical element in the production of groundnut with sound mature kernels is calcium (Meena and Malarkodi 2007). Calcium is an immobile element in phloem. Calcium moves upward (xylem) in the peanut plant, but does not move downward (phloem). Thus, calcium does not move through the peg to the pod and developing kernel. (Webb and Hansen 1989). Skelton and Shear (1971) stated that calcium is poorly translocated via the phloem of the gynophores and must be absorbed by the developing pod. However, groundnut crop is mostly cultivated in rain-fed conditions, where moisture cannot be assured at the time of gypsum application, continued to suffer due to calcium deficiency. Hence calcium application through foliar nutrition is research priority. Any element at nanoscale amenable for change in property specially increased surface area which enhances biological reactivity in plant cells. Calcium at nanoscale is presumed to acquire enhanced properties in a biological system including its mobility (Prasad et al. 2012).

The aim of this study was to test whether nanoscale calcium oxide particles can transport through phloem where the mobility of the bulk form is restricted and further to reveal that foliar application of nanoscale oxide particles could be used as one of the best calcium nutrient sources for the agricultural crops. To the best of our knowledge, this is the first report on evidenced phloem transport of nanoscale calcium oxide in groundnut.

Materials and methods

Experimental details (hydroponics)

The experiment was conducted in solution culture in a glass house at the Department of Crop Physiology, S.V.Agricultural College, Acharya N G Ranga Agricultural University, Tirupati. The groundnut seedlings (var. Narayani) were raised on acid-free quartz sand which was transferred to plastic troughs containing Hoagland solution on the eighth day after sowing. Aeration of the seedlings was regularly maintained by bubbling air into the nutrient solution by using an aquarium pump and without any damage to the root system. The experiment consists of eight treatments, viz., (1) Control (+Ca), (2) Bulk $CaNO_3$ 0.1 %, (3) Bulk CaO 0.1 %, (4) Nano-CaO 10 ppm, (5) Nano-CaO 50 ppm, (6) Nano-CaO 100 ppm, (7) Nano-CaO 500 ppm, (8) Nano-CaO 1,000 ppm.

Sowing

The quartz sand was thoroughly washed with normal tap water followed by overnight soaking of the sand in 5 % HCl. Then sand was washed with distilled water (DW) to make the sand acid free. Later sand was washed with double distilled water (DDW) for several times again. Groundnut Narayani seeds were sown in trays containing acid-free sand and seedlings were irrigated with double distilled water up to 7 days.

Preparation of solution culture

The culture solution was prepared in double distilled water following the dilutions of the stock solution and was stored in 1-L plastic beakers. Aeration to the root was provided by

bubbling air into the solution using an aquarium air pump. The solutions were changed every week.

Cement planks with a middle hole for plant and a side hole for the aeration tube was used as the lid for the beaker. The seedlings were carefully introduced through the hole and fixed with a pad of cotton so as the roots were completely immersed in the solution. The seedlings received half strength nutrient solution for the first week and later full strength solution. The solution level in the beakers was maintained by adding distilled water everyday. The plants were raised for 25 days and sampled. All the containers were randomized to reduce positional effects with three replications. The experiment was conducted in a glass house having a mean day and night temperature of 30 and 25 °C, respectively.

Nutrient composition

The composition of nutrient solution is given in Table 1. The nutrient solution used is a modified form of Hoagland formula (Johnson et al. 1957). All the reagents were of analytical grade and were used without further purification. 15 days after transferring to Hoagland solution, calcium deficiency symptoms developed. The treatments were imposed after noticing the deficiency symptoms. The composition of nutrient solution is given in Table 1. The nutrient solution used is a modified form of Hoagland formula. The developmental sequence of visual deficiency symptoms were carefully observed and documented. Calcium content was quantified in plant parts treatment-wise after terminating the experiment.

Table 1 Composition of Hoagland solution for solution culture technique

Compound	Molecular weight	Concentration of stock solution (M)	Concentration of stock solution (g l^{-1})	Volume of stock solution per liter of final solution (ml)	Final concentration of element		
					Element	µl	ppm
Macronutrients							
KNO_3	101.10	1.00	101.0	6.0	N	16,000	224
Ca $(NO_3)_2.4H_2O$	236.16	1.00	236.16	4.0	K	6,000	235
$NH_4H_2PO_4$	115.08	1.00	115.08	2.0	Ca	4,000	160
Micronutrients							
KCL	74.55	50	3.728	1.0	Cl	50	1.77
H_3BO_3	61.84	25	1.546		B	25	0.27
$MnSO_4H_2O$	169.01	2.0	0.338		Mn	2.0	0.11
$ZnSO_47H_2O$	287.55	2.0	0.575		Zn	2.0	0.131
$CuSO_45H_2O$	249.71	0.5	0.125		Cu	0.5	0.032
H_2MoO_4(85 % MoO_3)	161.97	0.5	0.081		Mo	0.5	0.050
Fe-EDTA	–	–	–	1.0	Fe	–	5.00

Stock solution (1 M)	ml/µl											
	Complete	–N	–P	–K	–Ca	–Mg	–S	–Fe	–Zn	–Mn	–B	–Cu
KNO_3	6	–	6	–	6	6	6	6	6	6	6	6
$Ca(NO_3)_24H_2O$	4	–	4	4	–	4	4	4	4	4	4	4
NH_4 H_2PO_4	2	–	–	2	2	2	2	2	2	2	2	2
$MgSO_47H_2O$	1	1	1	1	1	–	–	1	1	1	1	1
Fe-EDTA	1	1	1	1	1	1	1	–	1	1	1	1
Micronutrients	1	1	1	1	1	1	1	1	1 (–Zn)	1 (–Zn)	(–B)	(–Cu)
$NaNO_3$ (MW:85.00)	–	–	–	6	8	–	–	–	–	–	–	–
$MgCl_2.6H_2O$(MW:95.23)	–	–	–	–	–	–	1[d]	–	–	–		–
Na_2SO_4 (MW:142.05)	–	–	–	–	–	1[e]	–	–	–	–	–	–
$CaCl_2$ (MW:110.99)	–	4[a]	–	–	–	–	–	–	–	–	–	–
KCl (MW:74.55)	–	6[a]	–	–	–	–	–	–	–		–	–
$NaH_2PO_42H_2O$ (MW:156.01)	–	2[b]	–	–	–	–	–	–	–	–	–	–
NH_4Cl (MW:53.46)	–	–	2[c]	–	–	–	–	–	–	–	–	–

Preparation of Fe-EDTA: dissolved 26,1 g EDTA in 286 ml of 1 N KOH. Mixed this with 24.9 g $FeSO_47H_2O$ and diluted to 1 L before aeration. 1 ml of this solution provided 5 ppm in 1 L

[a] 355 ppm of Cl, [b] 45 ppm of Na, [c] 71 ppm of Cl, [d] 35 ppm of Cl, [e] 23 ppm of Na

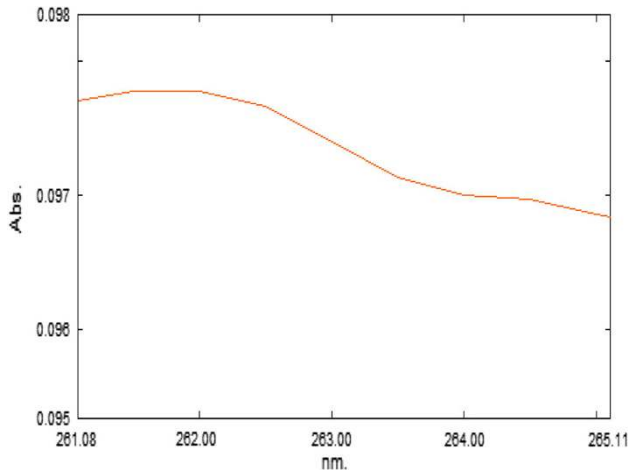

Fig. 1 UV–Vis micrograph showing typical absorbance (262 nm) of chemically synthesized n-CaO

Preparation of nanoscale calcium oxide (n-CaO) particles

Nanoscale calcium oxide particles (n-CaO) were prepared using sol–gel method. 1 % of calcium nitrate (Tetrahydrate purified LR, Sd-fine chemicals Ltd, Mumbai, India) was mixed with 0.05 % of sodium citrate tribasic dehydrate (extra pure AR, Sd-fine chemicals Ltd, Mumbai, India) and stirred at 60 °C for 3 h. Then the solution was filtered using filter paper (Whatman no. 1) and dried at 100 °C for 6 h. The collected powder was used for further characterization and experimental studies.

UV–Vis spectroscopic analysis

Solution of n-CaO was prepared by mixing 0.1 gm of n-CaO powder in 100 ml of water and stirred for 10 min. Then the solution was taken into 3 ml cuvette (Quartz) and scanned between 200 and 1,100 nm using the UV–Vis spectrophotometer (Shimadzu, UV-2450) and recorded the characteristic absorbance of the n-CaO.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) samples were prepared by pipetting the drops of calcium hydrosol on 200-mesh carbon-coated copper grids and allowing sample to dry in air. TEM micrographs were obtained using a Jeol 2010F HRTEM operating at 200 keV.

Particle size and zeta potential measurements

The hydrodynamic radius of the hydrosols is measured using the dynamic light scattering (DLS) technique wherein the scattering angle of laser light from the hydrosol is recorded and measured as size. The zeta potential, electrophoretic mobility and viscosity of the hydrosol were also measured using Nanopartica SZ-100 (HORIBA).

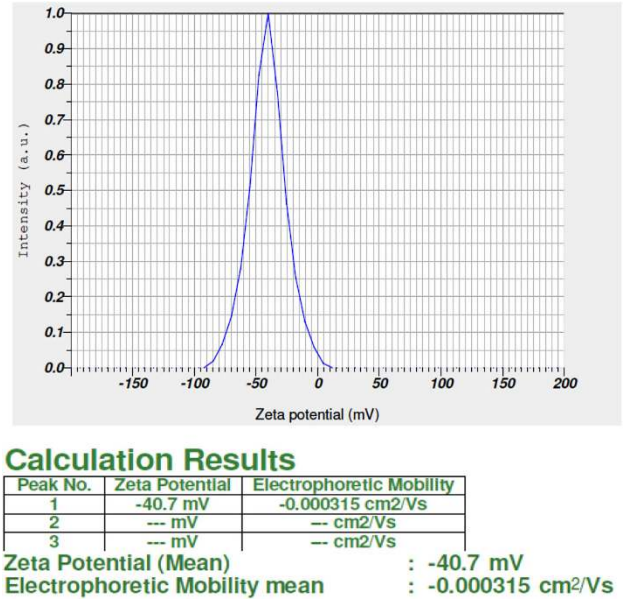

Fig. 2 Micrograph showing a negative zeta potential (−40.7 mV) of the n-CaO particles

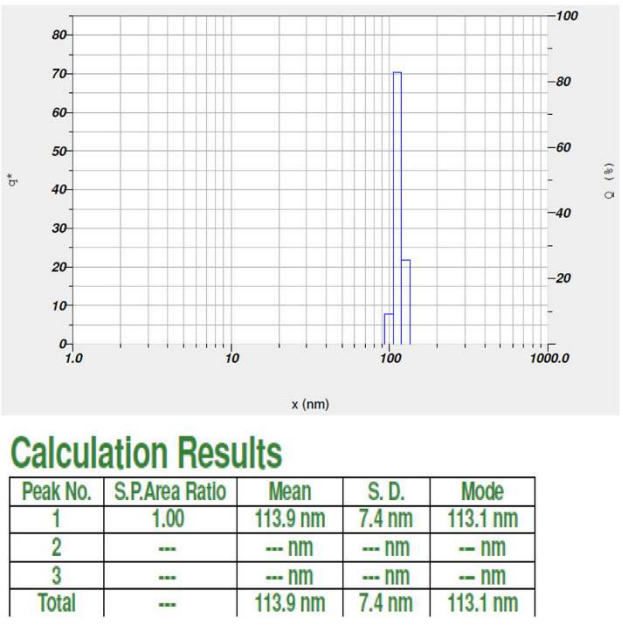

Fig. 3 Histogram showing the particle size distribution and hydrodynamic diameter of the n-CaO particles

Statistical analysis

The experimental data were analyzed statistically by following standard procedure outlined by Panse and

Sukhatme (1985). Significance was tested by comparing "F" value at five percent level of probability. Correlation studies were undertaken for different parameters of growth analysis, yield attributes and biochemical parameters according to the method proposed by Fisher and Yates (1963).

Results and discussion

The UV–Vis spectrograph showing the typical characteristic absorbance (262 nm) of n-CaO (Fig. 1). The broadening of the absorbance spectra indicates that the n-CaO particles are highly dispersed with uniform crystalline size. The high zeta potential value of (−40.7 mV) n-CaO also confirms that the particles are dispersed with strong electrostatic interaction forces (Fig. 2). The measured hydrodynamic diameter using the dynamic light scattering technique was 113.1 nm (Fig. 3). The relatively higher size of the hydrosol may be due to the bulging effect. Spherical and agglomerated calcium oxide nanoparticles were observed in TEM micrograph (Fig. 4). The agglomeration of the particles is due to the absence of protecting ligands on the surface of the particles. In the micrograph, the measured average size of the particles is 30 nm. Thus, the characterization of the n-CaO revealed that the particles are highly stable with well-defined size and shape.

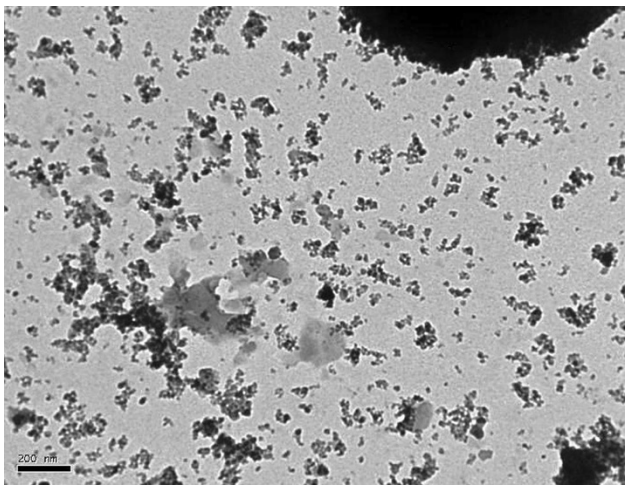

Fig. 4 Transmission electron microscopic view (200 nm-bar) of the calcium nanoparticles with mean size 30 nm

Since plants are unable to utilize calcium from old leaves, deficiency normally occurs first in the growing points and youngest leaves. Roots are usually affected before the tops, with both roots and tops exhibiting die back of the growing point. Where calcium deficiency is moderate to acute, root growth is markedly impaired and plants become susceptible to root-rot infection. Visual observations (Fig. 5) were made in development of deficiency and rectification in leaves and roots before and after spraying of different concentrations of nano-CaO, 0.1 % $CaNO_3$. Calcium content in different parts of the plant was analyzed and presented in Table 2.

Roots

Before spraying, relatively higher calcium percent (63–72 %) was recorded in the roots of control plants compared to calcium-deficient plant roots. After spraying of n-CaO and bulk calcium, significant differences were found between the treatments. After spraying of n-CaO at different concentrations (Fig. 6) and bulk calcium sources, calcium percent in the roots of deficient plants was significantly increased by 57.1–62.5 % except in the treatment sprayed with bulk CaO 0.1 %.

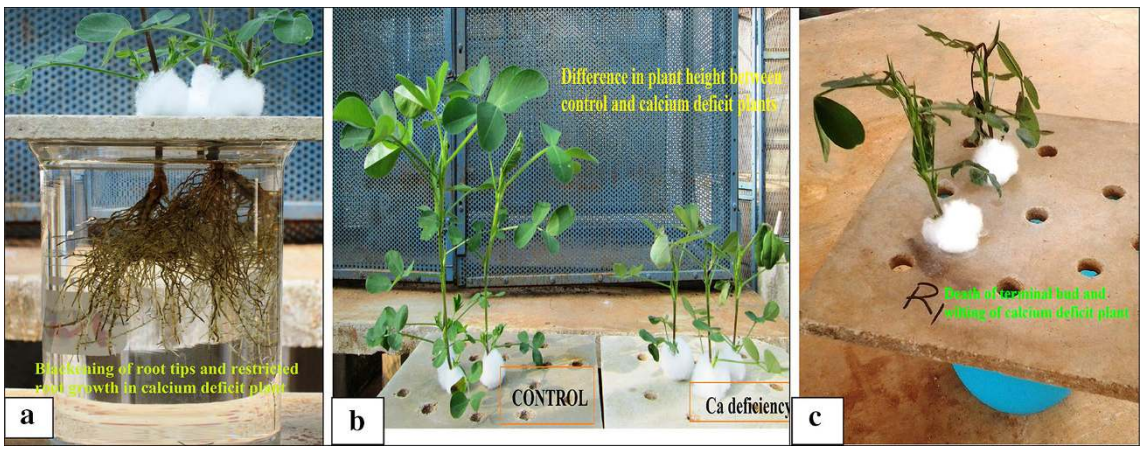

Fig. 5 Typical symptoms of calcium deficiency. **a** Blackening of the root tips with the restricted root growth in calcium-deficient plant. **b** Difference in plant height between control and calcium-deficient plants and **c** death of terminal bud and wilting of calcium-deficient plant

Stems

Prior to imposing of the treatments, control plant stems recorded highest calcium percent (80 %) compared to calcium-deficient plant stems. After spraying of n-CaO at different concentrations and bulk calcium sources, calcium percent in the stems of deficient plants was significantly increased by 77.7–81.8 %, except in treatment bulk CaO 0.1 %. Among the n-CaO treatments, 500 ppm recorded highest calcium percent in stems which is 36.36 % higher than control plant, 18.18 % than $CaNO_3$ and 27.27 % than bulk CaO. The concentrations of 100 and 1,000 ppm of n-CaO showed on par with 500 ppm, but both concentrations showed 9 % less calcium percent than 500 ppm. Ca^{2+} is transported in plant xylem vessel in chelate form and the speed of water flow is the key factor in Ca^{2+} transport via xylem in stem. There are both apoplastic and symplastic pathways of Ca^{2+} transport in fruit or leaf tissue too (Hong-Qiang and Yu-Ling 2005).

Table 2 Partitioning of calcium content (%) in various parts of groundnut in response to foliar spray of nano- and bulk calcium under hydroponic conditions

Treatments	Before spraying			After spraying		
	Root	Stem	Leaves	Root	Stem	Leaves
Control	0.011	0.010	0.010	0.011	0.007	0.005
$CaNO_3$ 0.1 %	0.004	0.002	0.002	0.007	0.009	0.011
Bulk CaO 0.1 %	0.002	0.002	0.002	0.005	0.008	0.011
Nano-CaO 10 ppm	0.003	0.002	0.002	0.007	0.009	0.011
Nano-CaO 50 ppm	0.004	0.002	0.002	0.008	0.009	0.011
Nano-CaO 100 ppm	0.003	0.002	0.002	0.008	0.010	0.012
Nano-CaO 500 ppm	0.003	0.002	0.002	0.008	0.011	0.013
Nano-CaO 1,000 ppm	0.003	0.002	0.002	0.008	0.010	0.012
	0.004	0.0030	0.0030	0.0077	0.0091	0.0107
SE ± m	0.00	0.00	0.00	0.00	0.00	0.00
CD 5 %	0.001	0.00	0.00	0.001	0.001	0.001
CV	9.337	8.472	5.705	5.979	5.648	5.292

Leaves

Before spraying of treatments, control plant leaves recorded highest calcium percent (80 %) compared to calcium-deficient plant leaves. After spraying, among the n-CaO treatments, plants treated with 500 ppm concentration of n-CaO recorded highest calcium percent in leaves which is numerically 61.53 % higher than control plants, 15.38 % than $CaNO_3$ and 15.38 % than bulk CaO. After spraying of n-CaO at different concentrations and bulk calcium sources, calcium percent in the leaves in deficient plants was increased by 81.8–84.6 %. However, foliar spray of bulk CaO 0.1 % did not show any increment in the calcium content in leaves, root and stems which further establishes that no evidence of calcium transport through phloem. The n-CaO concentrations of 100 and 1,000 ppm were shown to be on par with 500 ppm, but both concentrations showed 7.61 % less calcium percent than 500 ppm.

Ca^{2+} can enter cells passively through ion channels, but requires energy to be pumped out of the cytoplasm. The electrochemical potential for Ca^{2+} to enter the cytoplasm, across the plasma membrane was −52 kJ/mol (Spalding

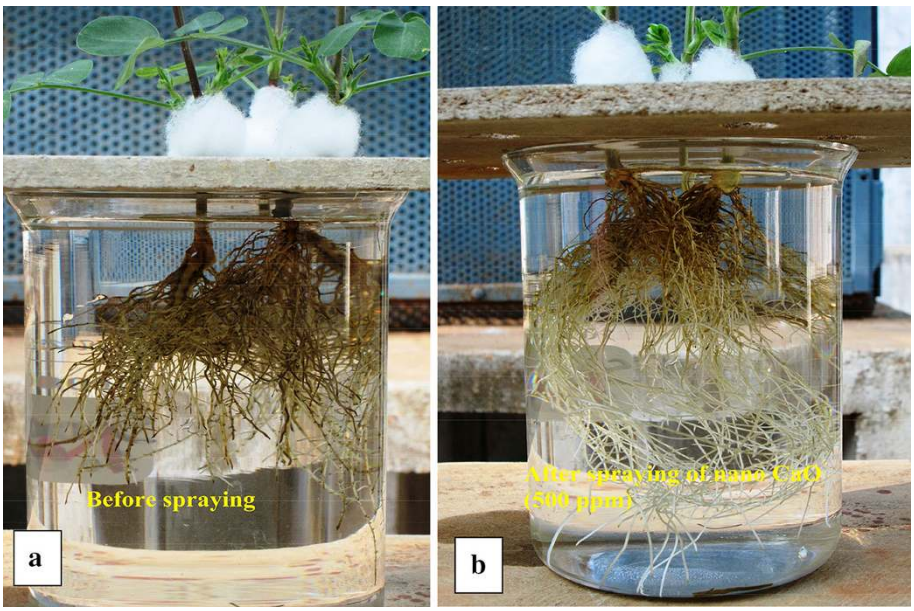

Fig. 6 Correction of calcium deficiency in groundnut with the application of 500 ppm of nanoscale calcium oxide nanoparticles (30 nm) in solution culture experiment: **a** before spraying. **b** After spraying of nano-CaO (500 ppm)

2011). Often, cytosolic Ca^{2+} levels fluctuate and they are in rapid control of membrane-localized Ca^{2+} pumps and channels located in plasmalemma, vacuoles and endoplasmic reticulum of plants (Stael et al. 2011; Ng and Mcainsh 2003; Yang and Poovaiah 2003). Therefore, it is anticipated that due to the small size, high surface area-to-volume ratio and continuum in flux, nanoscale calcium oxide particles could be transported with ease through ionic channels in the plant system and also we imagine that the relatively high electrochemical potential energy of the nanoscale materials also plays an important role in phloem transport of calcium oxide nanoparticles.

Conclusion

Phloem transport of nanoscale calcium oxide particles in groundnut using solution culture technique was confirmed for the first time which was an important breakthrough in agriculture and plant calcium nutrition. Calcium deficiency was well corrected by the application of nanoscale calcium oxide particles compared to their bulk counterparts (bulk CaO and $CaNO_3$). Our experiments also once again verified and confirmed the restricted mobility of bulk calcium in phloem. Germination and growth of groundnut significantly increased with the foliar application of nanoscale calcium oxide particles (average particle size 30 nm) and a dose of 500 ppm is proved to be effective. Thus application of nanoscale materials in agriculture is promising and efficient translocation of the nutrients to the desired plant parts could be achieved, grains in particular, which is an utmost important outcome in human health perspective.

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