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BOTANY

Impact of nano calcium hydroxide and calcium oxide on some metabolic activities and phenolic compounds of *Lupinus termis* seedlings

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Abstract

The effect of 0.5, 1.0 and 2.0 mM nano calcium hydroxide $Ca(OH)_2$ and calcium oxide CaO or bulk calcium carbonate $CaCO_3$ on growth and metabolic changes of *Lupines termis* seedlings was assessed. Root fresh and dry weights were increased by nano CaO at 0.5 and 1.0 mM compared with the control, while they were decreased by increasing nano $Ca(OH)_2$. Shoot fresh and dry weights were increased by increasing the concentration of nano $Ca(OH)_2$ and CaO reaching their maximum at 0.5 and 2.0 mM, respectively. Bulk $CaCO_3$ concentrations increased root and shoot fresh and dry weights at the highest concentration (2mM). The photosynthetic pigments (chl.a, chl.b and carotenoids) were markedly increased with increasing the concentration of nano Ca $(OH)_2$, nano CaO and bulk $CaCO_3$ with the highest magnitude recorded for nano $Ca(OH)_2$, while the lowest was for $CaCO_3$. The total soluble carbohydrate biosynthesis of both shoot and root was reduced by nano calcium particles, whereas it was enhanced by bulk calcium compared with control. The root soluble protein was decreased by different concentrations of either nano or bulk calcium, while shoot soluble protein was increased by all calcium treatments compared with the control. The nano calcium especially $Ca(OH)_2$ activated the protein metabolism compared with the bulk. The root and shoot phenolic compounds were decreased by different concentrations of either nano or bulk calcium, except of the shoot phenolic compounds at $CaCO_3$. Nano CaO at 0.5 mM increased shoot phenolic compounds by 10% compared with control.

Keywords: *Lupinus termis*, Nano calcium, Bulk calcium, Photosynthetic pigment, Growth criteria, Carbohydrates, Protein, Phenolic compounds.

INTRODUCTION

Lupinus termis (Lupin) plant is an herbaceous perennial plant belonging to the order fabales, family Fabaceae, subfamily papilionoideae (EFSA, 2005). The plant root is a normal tap root. The plant has a simple raceme inflorescence which produces as many as 70 flowers on the main stem. The flowers open in ascending order into cream orbicular or flattened shape pod.

The genus *lupinus* includes over 450 species worldwide (EFSA, 2005) but only the following four major cultivated species have gained agricultural importance viz., white lupin (*Lupinus albus* L.), blue lupin (*L.*

their physical and chemical characteristics. Nanoparticles (NPs) or (Nano scale particles= NSPs) are defined as ultrafine particles with lengths in two or three dimensions greater than 1 nm and smaller than 100 nm (ASTM, 2006). The physical and chemical properties of nanoparticles can differ significantly from those of bulk materials of the same composition. Due to NPs unique physical and chemical characteristics, recently they are widely used in many areas such as industry, agriculture, business, medicine, public health among many other (Rao and Shekhawat, 2014).

Ruffini-Castiglione and Cremonini (2009) have identified three types of NSPs: natural, incidental and engineered (can be carbon-based or metal based materials (Peralta-Videa *et al.,* 2011). Metal oxide is one of the metal- based groups. Metal oxide nanoparticles such as $Ca(OH)_2$ or CaO are important materials due to their widespread applications in various aspects including catalysis, sensors, optoelectronic materials, and environmental remediation (Oskam ,2006).

Calcium is the fifth most abundant element (by mass), usually found in sedimentary rocks in the mineral forms of calcite, dolomite and gypsum. It is one of the main macro essential nutrients for plant growth and development; formation of cellular walls, enzyme activators, metabolic processes, nitrate uptake, biomass accumulation (Savithramma, 2002) and photosynthetic rate (Savithramma, 2004; Savithramma *et al.,* 2007).

The aim of this study is to evaluate the effect of both nano calcium hydroxide and calcium oxide as well as bulk calcium on the growth and some metabolic activities of *Lupinus termis*

MATERIALS AND METHODS

angustifolius L.), yellow lupin (*L. luteus* L.) of the "Old World" or Mediterranean area lupin species and one "New World" species namely Andean lupin (*L. mutabilis* L.). Both of the last species have wild forms and cultivated crops (Gladstones, 1998; Erbas *et al.,* 2005) in Mediterranean region, Sudan, Ethiopia, Central and Western Europe, Australia, USA and South America, Tropical and Southern Africa, Russia, and Ukraine (Kwak *et al.,* 2000; Kettel *et al.,* 2003).

Plant seeds are high in protein, lipids, dietary fiber, carbohydrates and calcium (Gabrial and Morcos, 1976; Petterson, 1998 and Bhardwaj *et al.,* 2010). Lupin (quinolizidine alkaloid) is responsible for bitter taste and neurotoxins (Yeheyis *et al.,* 2011). The major *Lupinus albus* seed alkaloids are Lupanine (Huyghe, 1997; Olver, 1998; Getachew, 2009), hydroxyaphylline, albine, multiflorine (Getachew, 2009), anagraine (Yildiz, 2011) and sparteine (Huyghe, 1997; Erbas *et al.,* 2005).

Lupin has food applications, particularly as desirable additives in bakery and pasts products, or as meat, egg and milk replacers, traditional and alcohol production, snack as well as in dietary and functional food products (Loza and Lampart- Szczapa, 2008). Lupin protein with fibers lowers serum cholesterol level, improves glucose tolerance and consequently modifies blood insulin and glucagon (Chango *et al.,* 1998). The seeds are used as carminative, diuretic, emmenagogue, hypoglycemic, pectoral and vermifuge and are used as well as a poultice on ulcers. (Chiej, 1984).

The field of nanotechnology is one of the most active areas of research in modern material science. Nanoparticles exhibit completely new or improved properties based on their specific characteristics such as size, distribution and morphology affecting

fixed number of *Lupinus termis* seeds were sterilised by 0.01% $HgCl_2$ for one min, washed thoroughly with distilled water and divided into four groups. Each group was sown in plastic pots (15 cm diameter and 10 cm depth) filled with 5 kg clay-sandy soil (2:1 w/w) and 5 pots were used for each treatment. The first group of seeds was sown in untreated soil representing the control. The second one was sown in soil supplemented with different nano calcium hydroxide [Ca $(OH)_2$] concentrations (0.5, 1.0 and 2mM). The third group was sown in soil provided with different nano calcium oxide (CaO) concentrations (0.5, 1 and 2mM) and the seeds of the fourth one were sown in a soil supplemented with different calcium carbonate concentrations (0.5, 1.0 and 2 mM) and was considered as bulk $CaCO_3$. The seeds were left to germinated and grow as the usual practice and irrigated firstly with different concentrations of all calcium treatments to field capacity then irrigated with distilled water under greenhouse conditions. After 15 days of growth the seedlings were executed, washed with distilled water then separated into root and shoot. The growth criteria as lengths, fresh and dry weights of root and shoot were measured. Samples of three fresh leaves were kept frozen immediately for determination of the photosynthetic pigments. The remaining samples were dried in an oven at 60°C for determination of metabolic constituents.

Estimation of Photosynthetic Pigments:

The plant photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) were determined spectrophotometrically as recommended by Arnon, (1949) for chl. and Horváth *et al.* (1972) for

Synthesis of calcium oxide and calcium hydroxide nanoparticles

Calcium oxide (CaO) nanoparticles were prepared by calcination of $Ca(OH)_2$ which was synthesized by adding NaOH solution to $CaCl_2.2H_2O$ solution without using any surfactant, organic medium and complicated tools (Mirghiasi *et al.,* 2014), according to the reactions illustrated in equations. (1) and (2).

$$CaCl_2 + 2NaOH \rightarrow Ca(OH)_2 + 2NaCl$$

$$Ca(OH)_2 \xrightarrow{Calcination} CaO + H_2O$$

This method can be used as a simple, cheap and convenient way for producing calcium oxide and hydroxide nanoparticles on a large industrial scale.

Characterization of nano calcium

XRD Analysis

The crystalline structure of $Ca(OH)_2$ and CaO nanoparticles was characterized by X-ray diffraction (XRD, Shimadzu 6000 system, λ = 1.54 A˚).

TGA Analysis

Thermal behavior for the precursor was studied through thermogravimetric analysis (TGA), which was performed with a Shimadzu DTA- SO under nitrogen flow.

TEM Analysis

The morphology and average particle size of nano-particles were further investigated by a transmission electron microscope (TEM) (JEOL-Jem-2100).

Plant materials

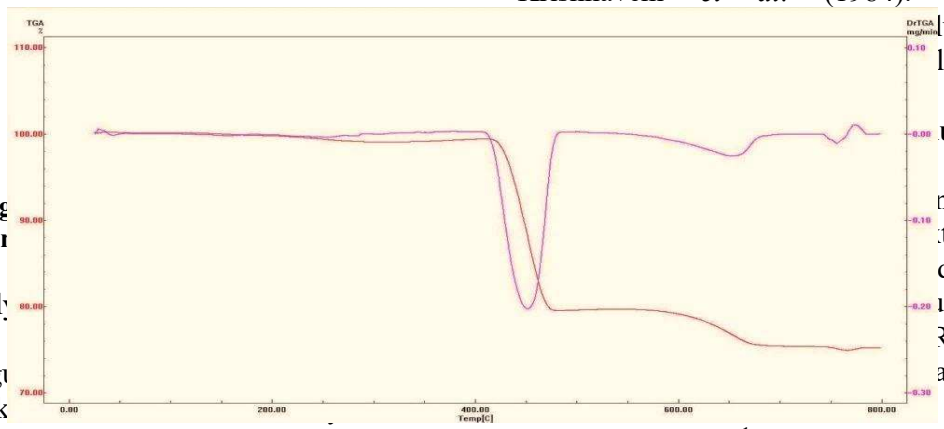
The experimental plant used in the present investigation was *Lupinus termis* (Lupinus) cv. Giza 1. Seeds were obtained from the Egyptian Ministry of Agriculture, Giza, Egypt**.**

Experimental Design:

During the growth season (November and December, 2014), a

Results

Nano particles were analyzed using different techniques to determine their physical nature. Thermogravimeteric analysis (TGA) showed two weight losses from 375 to 480℃ and 480 to 650℃ (Figure 1). These losses of weight were due to the decomposition of $Ca(OH)_2$ to CaO and the decomposition of $CaCO_3$ to CaO, respectively.



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$Ca(OH)_2$ and CaO were 3 peaks. The 2θ values of the strongest three peaks of crystalline $Ca(OH)_2$ were 18.14, 37.19 and 47.20°, whereas the 2θ values of the strongest three peaks of crystalline CaO were 18.09, 34.17 and 37.46°. The average crystallite size of $Ca(OH)_2$ and CaO nano-particles were calculated using Scherrer's equation (Eq. 3) to be about 73 and 91 nm for $Ca(OH)_2$ and CaO, respectively .

$$D=\frac{0.9\lambda}{\beta \cos} \qquad (3).$$

Where D is the mean crystalline size (nm), λ is the wavelength of Cu Kα (0.154 nm), β is the full width at half maximum intensity (FWHM) in radian and θ is the Bragg angle (°).

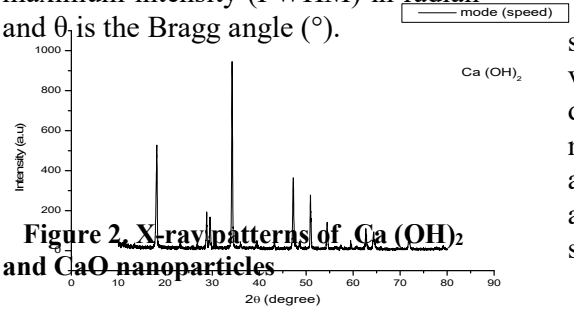

Figure 2. X-ray patterns of Ca (OH)$_2$ and CaO nanoparticles

carotenoids as adopted by Kissimon (1999). Chlorophyll concentration was calculated as mg g^{-1} dry weight of leaves.

Estimation of total soluble carbohydrates:

Carbohydrate extraction and clarification of plant materials (root and shoot) was performed according to Naguib *et al.* (1968). Total soluble carbohydrates content was estimated by the phenol sulphuric acid method described by Dubois *et al.* (1956) and Krishnaveni *et al.* (1984). The

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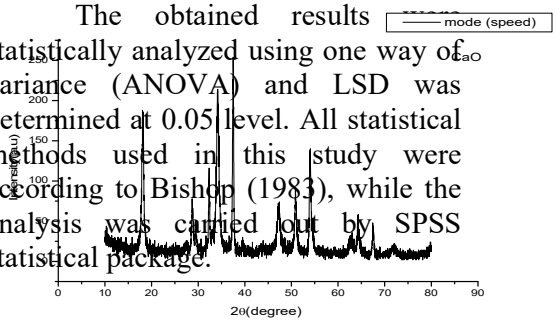
Quantitative estimation of total phenolic compounds

Extraction of total phenolic compounds was carried out according to Velioglu *et al.* (1998) and aqueous methanol extract of total phenolic compounds was determined according to the Folin Ciocalteu's method using spectrophotometer (Model RAY LIGHT UV- 9200). A standard curve was prepared using different concentrations of gallic acid and results were expressed as mg g^{-1} dry weight.

Statistical analysis:

The obtained results

statistically analyzed using one way of variance (ANOVA) and LSD was determined at 0.05 level. All statistical methods used in this study were according to Bishop (1983), while the analysis was carried out by SPSS statistical package.

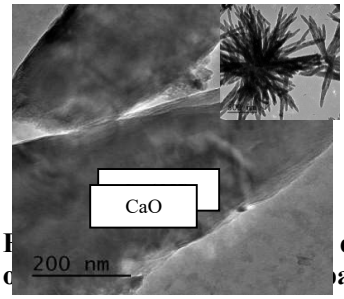


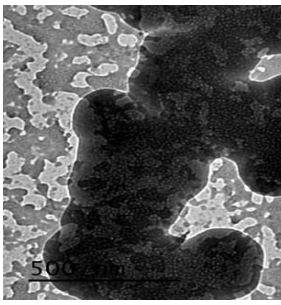
root dry weight was slightly increased by increasing concentration of nano $Ca(OH)_2$, with the exception of 11.5% decline at 1.0 mM nano $Ca(OH)_2$ treatment. It is clear that nano $Ca(OH)_2$ markedly increased both shoot fresh and dry weights by 34.0 and 29.0% respectively at its highest concentration (2.0 mM) compared with the control.

Application of nano CaO at concentrations of 0.5 and 1.0 mM high significant increased both root and shoot fresh and dry weights, but 2.0 mM nano CaO decreased both root and shoot fresh and dry weights relative to the control.

On the other hand, 0.5 and 2.0 mM bulk $CaCO_3$ increased both root and shoot fresh weights compared to the control. Root dry weight was gradually decreased by increasing concentration of bulk $CaCO_3$, with the exception of a slight increase in its dry weight by 2mM bulk $CaCO_3$ in comparison with the control. Shoot dry weight increased by bulk $CaCO_3$ at 0.5 and 2 mM, whereas it decreased at 1.0 mM $CaCO_3$ treatment compared to control.

The transmission electron microscope (TEM) micrograph of $Ca(OH)_2$ and CaO describe the nano particles structure (Figure 3). Through the TEM, nano $Ca(OH)_2$ and CaO had hexagonal and spherical shapes, respectively. The difference between XRD and TEM is that XRD shows the crystalline size of particles, while TEM shows their grain size of particles.





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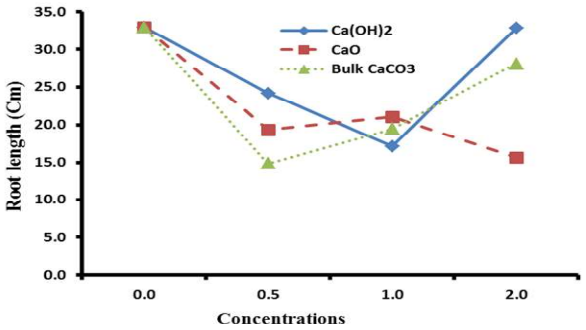
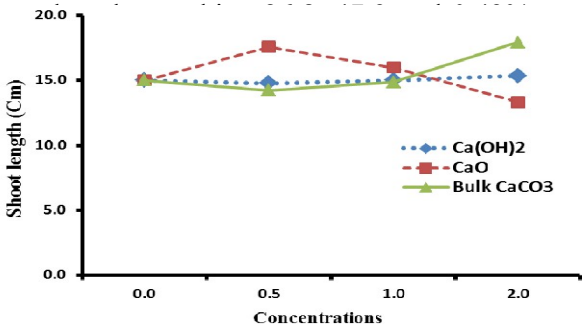
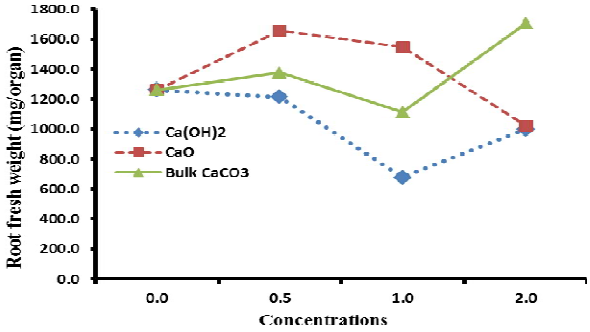
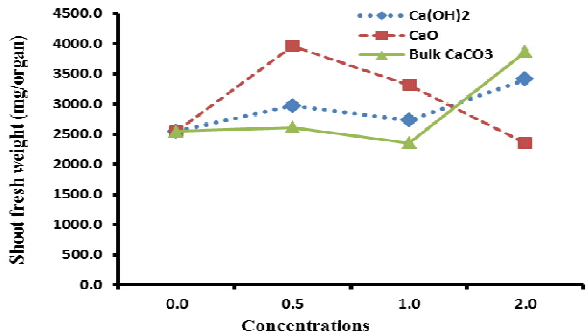
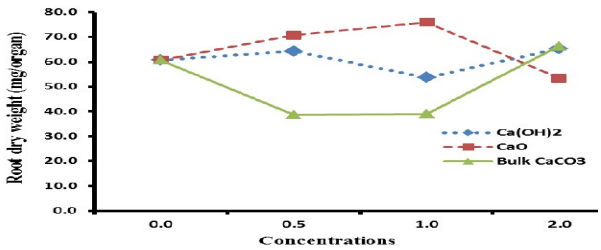
Regarding the effect of different concentrations of both nano $Ca(OH)_2$ and CaO and bulk $CaCO_3$, the results indicated that all treatments decreased the seedling root length in comparison with the control (Figure 4). The decrease was attained by bulk $CaCO_3$ treatment. Nano $Ca(OH)_2$ induced the least decrease in root

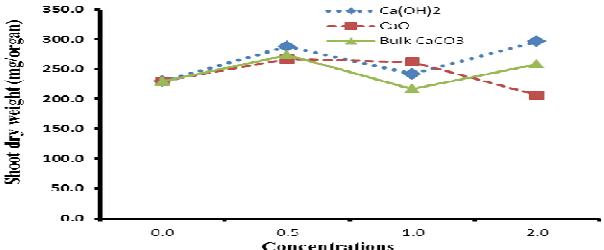










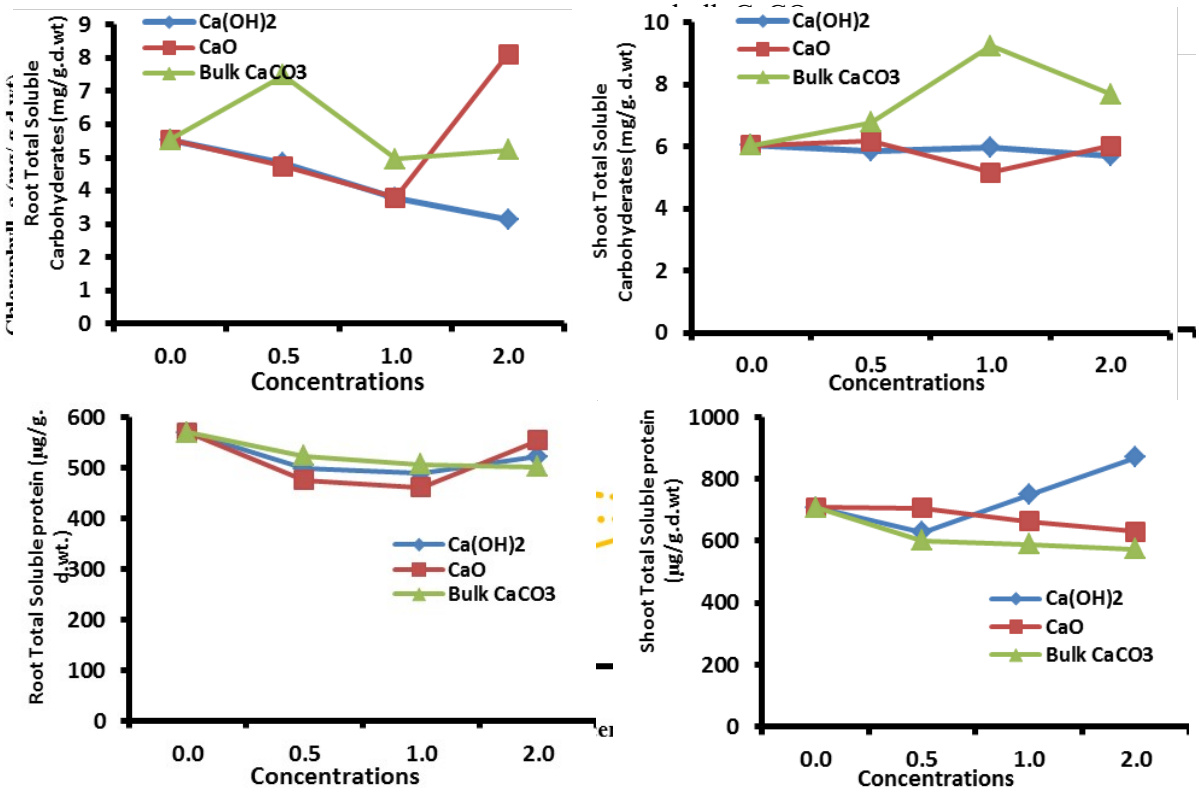


was gradually decreased in root by increasing the concentration of bulk $CaCO_3$, while it was increased in shoot reaching to 53.2% at 1.0 mM CaCO3 compared with the control.

It is evident from data in Figure 6 that total soluble protein content was decreased in root and shoot parallely to the increase in the concentration of both nano $Ca(OH)_2$ and CaO and bulk $CaCO_3$ with the exception of the shoot total soluble protein which was increased with nano $Ca(OH)_2$ at 1.0 and 2.0 mM compared with the control. The percentage of increase in the content of shoot total soluble protein at 1.0 and 2.0 mM nano $Ca(OH)_2$ was 5.8 and 23% , orderly compared with the control.

(mg/organ) of root and shoot of *Lupinus termis* seedling.

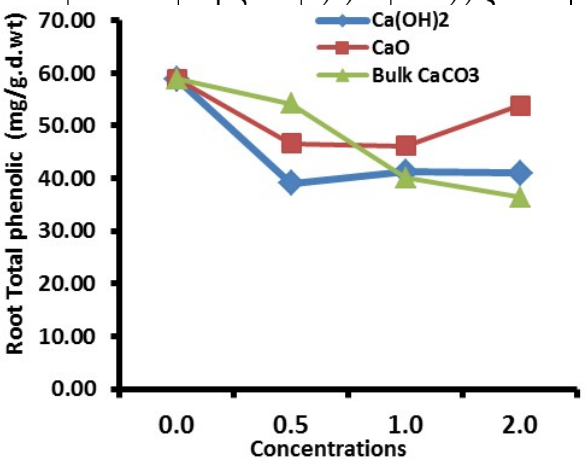
Application of both nano $Ca(OH)_2$ and CaO and bulk $CaCO_3$ induced pronounced increase in chlorophyll a, chlorophyll b and carotenoids with increasing their concentrations compared to control (Figure 5). The content of Chl.a, Ch.b and carotenoids was increased by nano $Ca(OH)_2$ reaching their maxima at 2.0mM where it amount to 188.0, 161.7 and 187.6%, respectively. The magnitude of increase by nano CaO was 152.4, 139,4 and 167.3%, respectively and by bulk $CaCO_3$ it reached 113.8, 118.7 and 92.2%, respectively compared with the control. This indicated that pigment biosynthesis was greatest by nano $Ca(OH)_2$ compared to their nano CaO

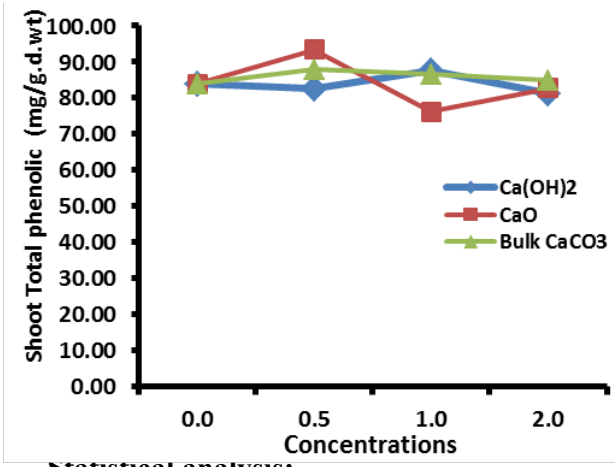


compounds was gradually decreased with increasing the concentration both nano calcium compounds and bulk $CaCO_3$ compared with the control. The magnitude of decrease was greater by both nano $Ca(OH)_2$ and CaO except for 2mM nano CaO which increased root total soluble carbohydrates by 46.4% relative to control (Figure 6). The content of total soluble carbohydrates

Table 1. Analysis of variance (ANOVA) for growth criteria, photosynthetic pigments and other metabolites under different concentrations of calcium forms and their interaction.

Parameters	Calcium		Interactions (Concentrations*Forms)
	Concentrations	Forms	
Root length	90.9**	15.2**	19.9**
Shoot	1.5**	2.2**	22.3**
Chlorophyll a content	167219.8**	6033.7**	8898.8**
Chlorophyll b content	5432.6**	183.5**	502.9**
Carotenoids content	353334.0**	56973.4**	25349.6**
Root carbohydrate	16175.2**	27469.1**	24144.5**
Shoot carbohydrate	4592.9**	49311.5**	13749.8**
Root protein	1972.9**	48.9**	272.8**
Shoot protei	4282.3**	27619.0**	12397.2**





nano compound at 0.5mM than by the bulk $CaCO_3$, while a reserve situation was observed at 1.0 and 2.0mM.

On the contrary, the content of shoot phenolic compounds was slightly increased at 1mM nano $Ca(OH)_2$ and at 0.5mM nano CaO and bulk $CaCO_3$ treatment achieving 4.3, 11.4 and 4.8%, respectively relative to the control.

Statistical analysis:

The statistical analysis (Table 1) indicated that different concentrations and forms of calcium applied to *Lupines termis* seeds led to highly significant variation ($P \leq 0.01$) in the seedling growth criteria, photosynthetic pigments and the investigated primary and secondary metabolites.

Savithramma (2013) who reported that shoot fresh and dry weights of *Vigna mungo* were increased by $CaCO_3$ nanoparticles as well as by 10 mM $CaCl_2$. In addition, TiO_2 nanoparticles were found to promote growth action of spinach (Yang *et al.,* 2006).

Application of high concentrations of both nano $Ca(OH)_2$ and CaO and bulk $CaCO_3$ induced pronounced increases in chlorophyll a, chlorophyll b and carotenoids relative to control. These results were in accordance with those obtained by Morteza (2013) and Tantawy *et al.* (2014) on *Zea mays* and tomato plants respectively. They reported that the content of chlorophyll (chl.a and chl.b) and carotenoids was significantly increased under nano TiO_2 spraying on the first plant and increased total chlorophyll content under 0.5 and 1.0 g/l nano calcium carbonate in the second one.

Total soluble carbohydrates content in root and shoot was significantly decreased by either nano calcium or $CaCO_3$. This result was consistant with that of Krishnaraj *et al.* (2012) who found that carbohydrates content of root and leaf of *Bacopa monnieri* were dropped in levels during subsequent exposure to nano silver and bulk silver nitrate. The concomitant increase in carbohydrates content in shoot with the decrease in root may indicate their translocation.

The results indicated that total soluble protein content was decreased in root and shoot parallely to the increase in concentration of both nano $Ca(OH)_2$ and CaO and bulk $CaCO_3$ s. These results agreed with those of Krishnaraj *et al.* (2012) who reported that the protein content in different organs of *Bacopa monnieri* plants treated with AgNPs was lower than that the of control.

Changes in total soluble carbohydrates and protein may be

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Root total phenolic	322258.1**	12.7**	11.2**
Shoot total phenolic	297393 9.6**	12.1**	57.0**

*** Significant at P≤ 0.05 ** highly significant at P ≤ 0.01**

Discussion

Very rare literatures were found about using nano calcium for improving plant growth and production (Liu *et al*., 2005). Results of the present study showed that calcium in the nano forms was superior in its effects compared with the control. However, both root and shoot lengths of *Lupinus termis* seedligs were significantly decreased with increasing all calcium treatments from (0.5 to 2.0 mM). These results agreed with those of Yin *et al.* (2011) who showed that Ag nanoparticles (AgNPs) has affected the root elongation and root growth of eleven wetland plants at very low concentrations. The present results are also in accordance with those obtained by Yasur and Usha Rani (2013) who found that using AgNPs (500- 4000 mg/l) did not affect shoot elongation patterns in the castor germinated seedlings. In addition, Yugandhar and Savithramma (2013) showed that shoot length of *Vigna mungo* was increased by the application of $CaCO_3$ nanoparticles. On the contrary, treatment of *Bacopa monnieri* by bulk $AgNO_3$ exhibited a marked retardation with increasing concentration (Krishnaraj *et al.,* 2012). In the present study, the significant increase in both shoot fresh and dry weights at 2 mM nano $Ca(OH)_2$ and 0.5 and 1.0 mM nano CaO was in agreement with results of Yugandhar and

attributed to changes in processes associated with photosystems, starch synthesizing machineries and/or carbohydrates translocation as postulated by Krishnaraj *et al.* (2012).

The biological molecules such as secondary metabolites could possibly play a major role in the synthesis and stabilization of the nanoparticles (Inbakandan *et al.,* 2010). In the present study, increased total phenolic compounds in the seedlings produced from seeds treated with both nano $Ca(OH)_2$ and CaO and bulk $CaCO_3$ showed a shift towards secondary metabolism. The content of root phenolic compounds was gradually decreased with increasing concentration of both nano calcium and bulk $CaCO_3$ compared with control. This result was consistent with Yasur and Usha Rani (2013) who found that the content of total phenolic compounds was decreased at 2000 mg/l of AgNPs in castor seedlings compared with control and all other treatments. The increase in the content of shoot phenolic compounds by 1mM of nano $Ca(OH)_2$ and by 0.5 mM nano CaO and bulk $CaCO_3$ may be referred to shoot allocation for such compounds rather than translocation to root.This was in harmony with the finding of Krishnaraj *et al.* (2012) who found that the total phenolic compounds in the leaves of *Bacopa monnieri* was increased by both forms of silver (AgNPs and $AgNO_3$).

It can be concluded that using calcium nanoparticles in the form of either $Ca(OH)_2$ or CaO is more beneficial for improvement of plant growth than using it as bulk $CaCO_3$.

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