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Effect of some rare earth elements on dry matter partitioning, nodule formation and chlorophyll content in *Arachis hypogaea* L. plants

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Abstract

Rare earth elements (REE) are found to be beneficial to plants in order to improve crop yield. The present study reveals the response of leguminous plant (*Arachis hypogaea* L.) to monazite soil containing Rare earth elements (Lanthanum and Samarium). This particular study explains the effects of rare earths on factors such as growth, total leaf chlorophyll content, number of root nodules and nitrogenase activity of groundnut plant. A significant increase in plant biomass and total chlorophyll content are observed. The increase in number of root nodules and the nitrogenase activity (Acetylene reduction, 20.72 n moles C_2H_4 formed / h/ g fresh nodules) were observed in the plants exposed to REEs. A profound effect of Lanthanum and Samarium present in the rare earth soil on the chlorophyll content, amylase activity, SOD and POD level were noticed.

Key Words: Nitrogenase, Chlorophyll, REEs,

Introduction

Rare earth elements (REEs) frequently occur together as rare earth minerals and have similarities in ionic radii and chemical activities (Henderson, 1984). Application of REEs in agriculture has been carried out intensively since 1972, aiming to increase crop yields (Brown et al., 1990; Xiong, 1995). Many research works have been done to show the beneficial effects of REEs on the synthesis of chlorophyll, seedling development (Chang, 1991; Wu et al., 1983) and stimulation of root and shoot growth in crops. Rare earth elements in plants induce metabolism and so they are used as fertilizer in agriculture. Metabolism of nutrients in plants increased by rare earth elements by the transfer of N from inorganic to organic form, which is beneficial for protein synthesis and regulation of nutrient balance (Yang and Zhang, 1986). Rare earth elements can enhance chlorophyll content and improve photosynthetic rate. Thus they can increase plant biomass. When an appropriate amount of rare earth elements was applied, the uptake of nutrients by plant and their transformation and utilization were promoted. Guo et al., (1988) Rare earth elements could enhance the plant's resistance against stress such as to cold, drought, acid rain and metal (Wen et al.,1992; Yan et al., 1999; Zhou et al., 1999) . Rare earth element in plants enhances the rate of respiration rate and decrease loss of water. Spraying rare earth on pepper foliars improved the total chlorophyll content of chlorophyll a and chlorophyll b (He et al., 1998). Many studies suggest that REEs can stimulate plants to absorb, transfer and assimilate nutrients.

Furthermore, both soil culture experiments (Zhu and Hu, 1988) and field trials (Zhu, 1992) demonstrate that enhanced N uptake by wheat plants after treating them with a mixture of rare earth nitrates, whereas Jie and Yu (1985) reported improved N utilization with in the range of 20.2 - 26.3 %. Rare earth elements applied to Chinese date trees increased the absorption of N and Zn. The application of fertilizer containing rare earth to rice plants, increased absorption of N by 16.4 % (Ning and Xiao, 1989). Additionally, sulfate absorption by soybeans was also enhanced. While seed dressing with rare earth nitrates has been shown to increase the contents of NO^{3-} in corn by 37.4 % (Cui and Zhao, 1994), decreased N contents were observed after the sole application of lanthanum (Diatloff et al., 1999). Alike, noncompetitive inhibitions of NO^{3-} uptake as well as reduced assimilation of NH^{4+} were observed in rice after the addition of lanthanum and cerium (Hu and Zhu, 1994). In contrast to that, increased absorption of nitrates was also reported in sugarcane (Kuang, 2006). The leaf nitrogen balance was decreased after the application of fertilizer containing rare earth nitrate. Additionally, an increase in total leaf nitrogen, a fractionation from nitrate to amino nitrogen and the free amino acid pool were observed (Liu and Wang, 2001). An accelerated transfer of N from inorganic to organic forms was also been described (Pang et al., 2002). This is considered to be beneficial for both the protein synthesis as well as the regulation of nutrient balance. Thus besides nutrient uptake, rare earths might also influence the metabolism of nutrients in plants. After the application of REEs, the number of root nodules increased

significantly, the activity of nitrogen-fixation was improved and the absorption of N by legumes was significantly enhanced (Wu et al., 1995). Enhanced nitrate reductase (nitratase) activity was noted in peanuts and tomatoes due to spraying of rare earth elements (Guo et al., 1988). Furthermore, after mixing seeds with rare earth nitrate reductase enhancements of 37 - 75 % was observed in leaves of winter wheat and the yield increased by 15.52 % (Yang and Zhang, 1986). The major objective of the present study is to determine the influence of REEs on *Arachis hypogaea* N metabolism and efficacy of REEs in root nodulation, photosynthetic growth and other enzymatic activities.

Materials and methods

Sample Collection

Rare Earth Element rich soil samples were collected from Chavara (Quilon District,Kerala,India). The samples were collected in sterile polythene bag and brought to the laboratory for analysis. The known weight of collected soil samples were acid digested with HCL / HNO_3 at a ratio of 3:1. It was made up to 10 ml with distilled water. Digested soil samples were analyzed for the evaluation of REEs concentration using ICP-MS (Perkin Elmer Sciex ELAN DRC II). The garden soil without traces of REEs was taken as control.

Plant material

Seeds of *Arachis hypogaea* L. were washed thoroughly with sterile distilled water thrice. The seeds were treated with 1% sodium hypochlorite. Then they were washed with distilled water to remove excess Sodium hypochlorite solution. The seeds were soaked in REE solution at a concentration of 10 mg/l (Indian monazite soil sample rich in REE), for 4- 8 hrs. The seeds were then pre-germinated. In the mean time polythene bags were prepared and folded pieces of germination paper inserted into the packet giving provisions for keeping the sprouted seeds on its top fold. The pouches were arranged in a wooden rack with iron rods meant for keeping the pouches upright. Three seedlings were kept in each pouch and a control was also run keeping the untreated pre germinated seeds in different pouches. Nitrogen free nutrient solution was added at the rate of 25 ml per pouch initially and 10-15 ml at weekly intervals. The plants were removed from the pouches after 30 days of sowing and the root system was observed for the presence and absence of nodules along with control.

Studies of various growth parameters, such as, Shoot length (Arts and Marks, 1971) and Root length (Buris et al., 1969) were done at 90 days interval with the following treatments.
(C- Control, T1- 10 mg/l monazite soil sample + seeds)

Chlorophyll extraction and quantification

Leaf chlorophyll content was measured following the method of Arnon (1949). Fresh leaf sample (0.5 g) was extracted and centrifuged at 3000 rpm for 10 min. The supernatant was collected separately and the pellet was re-extracted with 80 % acetone until the extract became colorless. All the supernatants were pooled and made up to a known volume with 80% acetone. Then the absorbance of the pigment was measured at 645 nm and 663 nm for Chlorophyll - a and Chlorophyll – b, respectively.

Determination of Amylase activity in seeds

Amylase enzyme was extracted from sprouted seeds with 0.1 N phosphate buffer (pH 6.7). The amount of product (maltose) formed was estimated, and the enzyme assay was carried out by DNS method (Palanivelu, 2001). 2.5 ml of 0.1 N phosphate buffer (pH 6.7) with 2.5 ml of 0.5 % starch solution, 0.5 ml of enzyme extracted from seed and 1 ml of water was mixed. The tube was incubated for 15 minutes. 0.5 ml of 2 N NaOH was added to arrest the enzyme activity and 3 ml of DNSA reagent (Dinitro Salicylic Acid) was added to the tube and kept in water bath for 15 minutes. The product formed (maltose) reacted with DNSA reagent and gave a colored complex. The optical density was measured at 540 nm using enzyme blank. The concentration of maltose produced was determined using standard graph for maltose

Estimation of SOD in leaves

Assay of SOD was based on the inhibition of formation of NADPH-phenazine methosulfate nitro blue tetrazolium formazon (Kakkar et al., 1984). The residual chromogen was extracted into an organic solvent like butanol. The colour formed at the end of the reaction was measured at 630 nm using a spectrophotometer as a measure of SOD activity. The leaves (0.5 g) were homogenized with 3 ml of potassium phosphate buffer (0.025 M pH 8.3) then the suspension was centrifuged at 5000 rpm for 10 minutes. 0.2 ml of the supernatant was mixed with 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of phenazine methosulfate (186 μM), 0.3 ml of Nitro blue tetrazolium (300 μM) and 0.2 ml of NaOH. The reaction mixture was incubated at 30°C for 90 seconds. Then the reaction was stopped by adding 1 ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4 ml of butanol and allowed to stand for 10 minutes. The reaction mixture was then centrifuged and the intensity of the chromogen in the butanol layer was measured at 560 nm against butanol as blank. The reaction mixture devoid of enzyme source served as control. One unit of enzyme activity is defined as the enzyme reaction that gives 50 % inhibition of NBT reduction in one minute.

Estimation of POD in leaves

In the presence of a hydrogen donor like pyrogallol or dianisidine, peroxidase converts H_2O_2 into water and oxygen. The oxidation of pyrogallol or dianisidine to a coloured product called purpurogalli can be measured using a spectrophotometer at 430 nm. An accurate amount of leaves (0.5 g) were homogenized in 2.5 ml of phosphate buffer (0.1 M). Then the mixture was subjected to centrifugation, and the supernatant was used as enzyme source. 0.2 ml of enzyme extract along with 3 ml of pyrogallol solution (0.05 M in 0.1 M phosphate buffer pH 6.5) was taken in a cuvette. 0.5 ml of H_2O_2 was added to the reaction mixture and the change in absorbance was recorded every 3 minutes. One unit of peroxidase activity is defined as the change in absorbance per minute at 430 nm (Reddy et al., 1985).

Table 1. REE analysis of soil sample by ICP-MS

S. No.	REE analyzed	Type of REE	Atomic Mass	Measured intensity mean	Concentration of the element Mean (ppm)
1.	Lanthanum (La)	Light	138.9055	785772.22	245.23
2.	Cerium (Ce)	Light	140.116	145331.34	175.60
3.	Samarium (Sm)	Light	150.36	53046.821	211.12
4.	Gadolinium (Gd)	Heavy	157.25	32570.642	40.81
5.	Neodymium (Nd)	Light	144.24	34027.163	10.80

Table 2. Assessment of biomass (*Arachis hypogaea L.)*

S.No	Treatment	Shoot fresh weight (g/plant)	Shoot dry weight (g/plant)	Root fresh weight (g/plant)	Root dry weight (g/plant)	Root length (cm)	Shoot length (cm)
1.	With REE	13.5 ± 0.43	3.8 ± 0.13	12.6±0.40	2.84±0.17	11.1 ±0.54	30.6±0.25
2.	Without REE	11.4 ± 0.06	2.84 ± 0.06	11.3±0.25	1.77±0.13	8.1 ± 0.74	22 ± 0.20

Values are mean ± SD of triplicates. Statistically not significant (P>0.05) compared to untreated seeds

Leonard jar experiment

Groundnut seeds were surface-sterilized and after 2 days of germination at 28°C in the dark, the seedlings were transferred to Leonard jars. Nitrogen-free Norris medium, pH 7.0 (Vincent, 1970) was used as the nutrient solution. The upper parts of the Leonard jars were filled with vermiculite, and the seedlings were treated with REE and then covered. The jars were provided with a filter paper strip and aluminum foil for root support. After treatment, the plants were grown for approximately 1month at 37°C during the day (16-h) and at 25°C during the night (8-h) period.

Root nodules quantification and assessment of Nitrogenase activity

The number of root nodules was enumerated 90 days after sowing by pulling out the plants carefully and by counting the number of matured root nodules with the help of hand lens. The nodules were separated from the root system and were weighed (in an electrical balance with 0.001 g capacity) and the fresh weight of the nodules per plant was expressed in grams (g). Then the root was dried at 70^{o}C and the dry weight of the nodules per plant also expressed in grams.

Nitrogenase activity was assayed by using the acetylene reduction technique (Stewart et al., 1968). Plants were harvested and roots were washed thoroughly to remove the adherent soil particles. Nodules were detached from the roots carefully without any damage and put in the test tubes (20 ml volume). The tubes were sealed with rubber serum stopper. Ten percent of air (2ml) was withdrawn from each tube and replaced with 2ml of acetylene. After 1 hr of incubation at 28±1^{o}C, 0.1 ml of gas was drawn from each tube and analyzed by a 5890 Hewlett-Packard Gas Chromatograph equipped with a flame ionization detector (FID) and propack T column with nitrogen as the carrier gas at a flow rate of 20ml/min. The other conditions were: Column temperature 75^{o}C, detector temperature 120^{o}C, and injection port temperature 110^{o}C. The quantity of ethylene production was calculated from peak areas of ethylene. Authentic ethylene was used as standard. The activity of nitrogenase was expressed as µ moles of ethylene formed as $h^{-1}g^{-1}$ fresh nodules.

Results and discussion

The soil sample collected from Chavara (Quilon District, Kerala, India) was analyzed for REE by ICP-MS (Table1). Lanthanum (La) and Samarium (Sa) were abundant in the soil sample (245.23 and 211.12 ppm respectively). Parthasarathy et al., in 1986, reported lanthanide contents of Indian monazite that had an appreciable range of composition in terms of REEs. The REE distribution of monazite shows the general enrichment of Light Rare Earth Elements which is due to preferential incorporation of LREEs relative to the Heavy Rare Earth Elements in monazites. In the present study, a similar type of distribution of LREEs especially La and Sm were found to be rich in the soil samples of Chavara. The communication by Jeya et al., (2000) also reported that the presence of REEs in monazite sample was comparatively higher than other elements in the samples of both Chavara and Manavalakurichi. Partitioning of plant biomass (fresh and dry weight) in root and shoot was evaluated, moreover the length of these organs were measured in both treated and control plants. It showed a significant increase in root and shoot length. The dry weight of root and shoot and fresh weight of root and shoot, treated with REEs were compared to other trials (Table 2).

Significant fractionation of La and Sm occurred in different parts of wheat plant such as root, shoot and leaves respectively (Table 3).

Table 3. Concentration of REEs in acid digested parts of plant by ICP –OES analysis

S. No.	Sample	REEs accumulation mg / L	
		La	Sm
1	root	5.01	7.08
2	shoot	2.1	4.9
3	leaf	1.1	2.7

Values obtained as such from ICP-OES analysis for La Lanthanum) and (Sa) Samarium.Ce (Cerium) was found to be below detectable limit (ppm / mg / L).

Table 4. Estimation of Chlorophyll (mg / g of fresh leaves) content in leaves of *Arachis hypogaea* L.

S.No.	Treatment	Chlorophyll a mg/g	Chlorophyll b mg/g	Total Chlorophyll mg/g
1	With REE	0.113 ± 0.02	0.361 ± 0.01	0.412 ± 0.01
2	Without REE	0.074 ± 0.02	0.296 ± 0.01	0.334 ± 0.01

Values are mean ± SD of triplicates. Statistically not significant (P >0.05) compared to untreated seeds.

Table 5. Study of root nodules (*Arachis hypogaea L.)*

S.No	Treatment	Number of root nodules / plant	Nodules fresh weight (g / plant)	Nodules dry weight (g / plant)	Nitrogenase activity (Acetylene reduction) n moles C_2H_4 formed / h/ g fresh nodules
1	With REE	33.7±0.01	0.177±0.02	0.052±0.02	20.72 ± 0.03
2	Without REE	15.3±0.01	0.128±0.02	0.020±0.02	15.40 ± 0.02

Values are mean ± SD of triplicates. Statistically not significant (P >0.05) compared to untreated seeds.

Table 6. Physiological parameters as affected by REEs in plant (*Arachis hypogaea L.)*

Parameters	With REE	Without REE
POD (U#/gm)	74.12 ± 1.08	68.32 ± 0.45
SOD (U$/gm)	15.02 ± 0.12	13.04 ± 0.11
Amylase*	2.89 ± 0.12	2.64 ± 0.11

*Enzyme Activity is expressed in units; 1 unit = 1 nmol Maltose utilized/ mg of protein /min. U# - unit = change in absorbance / min at 430 nm . U$ - unit = Activity of enzymes that gives 50% inhibition of the extent of NBT reduction in 1 min. Values are mean ± SD of triplicates. Statistically significant (P<0.05) compared to untreated seeds.

LREEs enrichment was observed in soil sample. Higher percentage of fractionations of REEs was noticed in seeds treated with high concentration of REEs. The effects of REEs on improving the absorption and mobility of nutrient elements depend on the methods used in treating the plants. In the present study, the ICP-OES analysis of the acid digested diverse parts of plants reveal that the accumulation of rare earth element is higher in roots than in other parts of plants. REE concentrations are elevated in roots and they decrease in the order of roots > shoot > leaves (Wyttenbach et al., 1998). Although at low concentrations, contents of individual rare earths in aerial parts of plant samples were similar between control and treated group ranking in the order of Ce > La > Nd > Pr > Sm; another concentration order of La > Ce > Nd > Pr > Sm was noticed when rare earths were applied at higher doses, clearly indicating the incorporation of exogenous rare earths into plant tissues (Wang et al., 2001b).

Greater biomass and increased root length were observed after the exposure to light rare earth elements such as Ce, Sm and La. It showed a remarkable variation in the total chlorophyll content of the leaves of *Arachis hypogaea* after exposure to lanthanides (Table 4). The bioavailability of nutrients and ions depend largely on the physico-chemical and biological characteristics of soils, especially the dynamic processes in the rhizosphere. The changes in the rhizosphere caused by the growth of roots could be an important factor causing the fractionations of REEs. Liu et al., (1999) reported that, Rare earth elements added to the soil were rapidly converted to other forms. A reduction in the soluble exchangeable fraction was observed. At the same time, rare earths complexed to organic matter and remained unchanged in the beginning then they increased but the residual rare earths remained stable. The influence of acid precipitation was studied by Chen et al., (1995). Secretions from the root system might contain some simple organic acids and amino acids which might promote desorption of REEs from the soil and also influence the REEs diffusion from soil to root by forming complexes with REEs (Shan et al., 2002). Xu et al., (2003) found that a sole application of lanthanum at relatively smaller doses compared to mixtures of rare earth elements results in a substantial accumulation of lanthanum in maize plants. This further supported the assumption that rare earth uptake by roots as well as the subsequent transport of the absorbed elements from the roots to the plant tops varies with each rare earth element.

The reduction of acetylene is higher in the presence of REEs than in the absence of REEs which infers enhanced nitrogenase activity by REEs. The nodules of *Arachis hypogaea* after exposure to lanthanides show an increase in the Nitrogenase activity, (Acetylene reduction, 20.72 n moles C_2H_4 formed / h/ g fresh nodules) when compared to the nodules of untreated seeds (Table – 5).REEs especially Lanthanum and Samarium affected the parameters monitored in the study (Table 6). Increased activity of enzymes like amylase in the presence of REEs may influence the coleoptile formation and biomass production (Xiong et al., 2000). The physiological responses include more chlorophyll, enhanced rate of development, greater production of roots and stronger tillering.

Although there is no clear evidence to prove that REEs are necessary for plants to grow, many studies suggests that REEs can stimulate plants to absorb, transfer and assimilate nutrients (Chang et al., 1998). The metabolism of nutrients in plants show an increased absorption of N, P, and K in rice by 16.4% 12%, and 8.5%, respectively. The absorption of sulfate by soybeans is also augmented after the application of REEs. These results suggest that the effects of REEs on improving the absorption of nutrient elements depend upon the methods used in treating the plants. Soaking of seeds with low concentration of REEs (1-2 g REE per kg seeds) than with high concentrations, show best effect in growth of plants (increased root weight of 10.9 - 34.6 % in soybean) (Xiong et al., 2000). At the same time, accelerated root nodule formation and nitrogen fixation were observed (Chen, 1991).

It has also been suggested after observation in China, that the increased photosynthetic abilities of several crop species may be attributed to the enhancing effects of rare earth elements (Wang et al., 2003a). However, mixed rare earth elements (La, Ce and Sm) were widely reported to be beneficial for plant photosynthesis and effects were obvious in numerous plant species. The total chlorophyll contents of chlorophyll a and b could be improved after a rare earth containing solution of 200 - 800 mg/l was sprayed on pepper (He et al., 1998). The principle of their enhancement on plant photosynthesis is probably related to enhanced enzyme activity, chloroplast development as well as increased chlorophyll contents. An accelerated photosynthetic light reaction as well as increased chlorophyll content was observed after the application of lanthanum chloride (Chen et al., 2001).

The number of nodules in the leguminous plants and the nitrogenase activity of the nodules were carried out, and those plants treated with soil containing REEs have profound effect and variation compared to the untreated and ethylene as control. The increased rate of acetylene reduction showed that the nitrogenase activity was consistently high in all plants treated in REEs enriched soil sample. The highest of the nitrogenase activity was recorded on the 90^{th} day which was significantly higher than those observed with untreated seeds and control (ethylene). Nitrogenase activity in peanuts and tomatoes was enhanced markedly by spraying REEs (Guo, et al., 1988). In addition, Yang and Zhang (1986) showed that the nitratase activity in the leaves of winter wheat was enhanced by 37-75% after blending seeds in REEs, and the yield was improved by 15.52% compared with control groups. La, Ce, and Pr of less than 50 mg/L could increase photosynthesis in nitrogen-fixation alga. Spraying 200-800 mg/L of REEs on pepper foliars improved the total chlorophyll content of Chlorophyll – a and Chlorophyll - b (He et al., 1998). REEs could also increase the assimilation of nutrients in seeds and in transport to plants (Liu and Wan, 1993).

Rare earth micronutrient fertilizer (REMF) could promote POD activity in tea shoots and the tea shoot biomass was enhanced. Wang et al., (2003) reported that the POD protein in tea shoots was found to bind rare earth elements, mainly La, Nd and Ce. Along with enhanced respiratory rate, Hong et al., (2000a) reported increased activities of superoxide dismutase, catalase, and peroxidase as well as decreased superoxide O^{-2} in rice seeds treated with lanthanum nitrate. It was furthermore demonstrated that rare earth elements may affect the activity of enzymes involved in antioxidant defense. Neodymium chloride was shown to increase the activity of superoxide dismutase (SOD) and peroxidase (POD), decrease the malonyldialdehyde (MDA) contents, reduce membrane demagnification and enhance the ability of plants to remove O^{-2} (Arnon and Chen, 1994). Significant increases in the contents of glucose and fructose were reported in sugar beet leaves after foliar application of rare earths. Since another study observed a decreased activity of the sucrose-transform enzyme (amylase) of 34.2 - 84.7 % after sugar beet plants were sprayed with 0.1 to 500 μg/l of rare earth (Bai and Chen, 1989), it was suggested that changes in enzyme activity account for increased sugar contents. Additionally, neodymium increased the activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) while decreasing the penetration of cell membranes. In certain plants (rape) the activity of peroxidase (POD) was shown to increase gradually with increasing concentrations of lanthanum (Zeng et al., 2001).

To conclude significant fractionations of La and Sm occurred in different parts of *A.hypogaea* plant such as root, shoot and leaves respectively. LREEs enrichment was observed in soil sample. Higher percentage of fractionations of REEs was noticed in seeds treated with high concentration of REEs (10mg/l). The effects of REEs on improving the absorption and mobility of nutrient elements depend upon the methods used in treating the plants. REEs have a profound effect on the chlorophyll content, POD and SOD level. Increased activity of enzymes like amylase and nitrogenase in the presence of REEs may influence the nodulation, coleoptile formation and biomass production.

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