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Role of foliar application of 24-epibrassinolide in response of peanut seedlings to iron deficiency

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Abstract

Limited information is available on the role of brassinosteroids (BRs) in response of plants to nutrient deficiency. To understand the functions of BRs in response to iron deficiency, we investigated the effect of 24-epibrassinolide (EBR) on activities of ferric-chelate reductase (FCR), H^+-ATPase, Ca^{2+}-ATPase, nitrate reductase (NR), antioxidant enzymes, Fe and other minerals content and distribution, chlorophylls, soluble protein, free proline, reactive oxygen species, and malondialdehyde in peanut (*Arachis hypogea* L.) plants subjected to Fe deficiency (10^{-5} M Fe(III)-EDTA) with foliar application of EBR (0, 10^{-8}, 5.0×10^{-8}, 10^{-7}, 5.0×10^{-7}, and10^{-6} M). Results show that EBR increased Fe translocation from roots to shoots and increased Fe content in cell organelles. Activities of antioxidant enzymes increased and so the ability of resistance to oxidative stress was enhanced. As result of enhancement of H^+-ATPase and Ca^{2+}-ATPase activities, the inhibition of Fe, Ca, Mg, and Zn uptake and distribution was ameliorated. Chlorophyll, soluble protein, and free proline content also increased and consequently, chlorosis induced by Fe deficiency was alleviated. The results demonstrate that EBR had a positive role in regulating peanut growth and development under Fe deficiency and an optimal concentration appeared to be 10^{-7} M.

Additional key words: antioxidant enzymes, brassinosteroids, chlorophylls, mineral elements, reactive oxygen species, subcellular distribution of Fe.

Introduction

Iron (Fe) is essential nutrient for most organisms. In plants, Fe participates in a series of biochemical processes such as photosynthesis, respiration, nitrogen fixation, electron transfer, and DNA synthesis, and involves in enzyme activation (Graziano and Lamattina 2007). Though Fe is the fourth abundant element in the earth crust, it often forms insoluble Fe (III) oxides, and therefore, its bioavailability is limited, especially in calcareous soils which account for 30 % of the world's cultivated soils (Mori 1999). Chlorosis induced by Fe deficiency is common nutritional disorder which affects a wide range of crops (Marschner *et al.* 1995). In addition to reducing crop productivity, Fe deficiency ultimately influences food quality and human nutrition (Zuo and Zhang 2011).

Under Fe deficiency, plants employ a set of responses to promote Fe mobilization and uptake, regulate iron internalization, translocation, and storage in cells (Curie and Briat 2003, Hell and Stephan 2003). Higher plants have developed two distinct strategies to enhance Fe availability. Strategy I plants, which are comprised of the dicots and nongrass monocots, are characterized by release of a proton (H^+), induction of Fe(III)-chelate reductase (FCR), and subsequent transport of a Fe (II) ion across the root plasma membrane (Kobayashi *et al.* 2003). Strategy II plants, which are represented by

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Abbreviations: ALA - aminolevulinic acid; BRs - brassinosteroids; Car - carotenoids; CAT - catalase; Chl - chlorophyll; CK1 - control; CK2 - low Fe treatment; EBR - 24-epibrassinolide; EBR1-5 - low Fe treatment combined with foliar application of various concentrations of EBR; FCR - ferric-chelate reductase; MDA - malondialdehyde; NR - nitrate reductase; $O_2^{\bullet -}$ - superoxide anion; PM - plasma membrane; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase.

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graminaceous species, acquire iron by exuding phytosiderophores (PS; Marschner *et al.* 1994). Besides, plants respond to Fe deficiency through morphological changes such as root hair branching, root-tip swelling, and lateral root extension to enhance Fe uptake (Schmidt 1999, Muller *et al.* 2004). However, when Fe availability is below a critical level, plants may suffer Fe deficiency with typical leaf chlorosis symptoms which occur in many crops, particularly peanut, cucumber, tomato, and soybean (Legay *et al.* 2012, Gonzalo *et al.* 2013, Kong *et al.* 2014b).

As environmentally-friendly class of plant hormones, brassinosteroids (BRs) have been widely applied to promote crop production under environmental stresses (Khripach *et al.* 2000, Krishna 2003, Fariduddin *et al.* 2014). Previous studies have indicated that BRs can affect a variety of physiological processes at nanomolar to micromolar concentrations (Sasse 2003) that modulate plant growth and development, including cell division and elongation, ethylene biosynthesis, senescence, photosynthesis, protein and nucleic acid biosyntheses, enzyme activation, and gene expression (Hayat *et al.* 2011). Therefore, BRs have been applied to improve plant growth and yield under drought (Sairam 1994, Fariduddin *et al*. 2009), salinity (Fariduddin *et al.* 2013), cold (Kagale *et al.* 2007), heat (Wilen *et al.* 1995), and heavy metal toxicity (Hasan *et al.* 2008, Fariduddin *et al.* 2009). Recently, application of BRs has been also reported to enhance CO_2 assimilation rate and carboxylation efficiency, and increase chlorophyll (Chl) and proline content and activity of reactive oxygen species (ROS) scavenging enzymes (Hayat *et al.* 2010, Fariduddin *et al.* 2013). It is hypothesized that BRs may ameliorate plant chlorosis induced by Fe deficiency.

Peanut is important oilseed crop in China, accounting for 30 % of the total oilseed production in the country. However, Fe deficiency-induced chlorosis represents a severe and common problem to peanut production on calcareous soils. Peanut employs a range of responses to Fe deficiency, including enhanced root branching and subapical root hair development (Chen *et al.* 2010), increased release of protons and reductants, such as phenolic compounds, into the rhizosphere (Jin *et al.* 2007), and induces both plasmalemma FCR and a plasmalemma Fe (II) transporter in root cells (Zhang *et al.* 2012). Despite these strategies, the chlorosis in peanut is still severe in calcareous soils. Application of inorganic Fe-compound fertilizers has a limited effect on plant chlorosis (Shenker and Chen 2005). Rodríguez-Lucena *et al.* (2010) showed that synthetic Fe-chelates (*e.g.*, Fe-ethylene diamine tetraacetic acid and Fe-ethylene-diamine-N,N'-disuccinic acid) are more efficient than inorganic Fe compounds [*e.g.*, $Fe_2(SO_4)\ .\ 7\,H_2O$] and natural Fe-complexes (*e.g.*, lignosulfonates and amino acids) for correcting Fe chlorosis in soybean, but they are usually expensive (Abadía *et al.* 2011). Therefore, further research is needed to alleviate plant chlorosis induced by Fe deficiency. The aims of this study were to investigate the role of different concentrations of 24-epibrassinolide (EBR) in response to Fe deficiency of peanut seedlings and to determine the optimal concentration of EBR for correcting peanut chlorosis.

Materials and methods

The experiment was conducted in the College of Resources and Environment of the Shandong Agricultural University. The seeds of peanut (*Arachis hypogaea* L.) were surface sterilized with 5 % (v/v) H_2O_2 for 30 min, washed with deionized water, and germinated on wet sterile sand at 25 °C in darkness. After germination, uniform seedlings were transferred into an aerated one-half strength Hoagland nutrient solution (Hoagland and Arnon 1950) for 3 d. The solution was subsequently exchanged with the full-strength Hoagland solution. When the third leaf expanded fully, cotyledons were removed and the seedlings were transferred into 3-dm^3 black plastic containers with different nutrient solutions (six seedlings per container). The nutrient solution was renewed every 3 d. The experimental design consisted of a control (the full-strength Hoagland solution; indicated as CK1), a low Fe content [the Hoagland solution with 10^{-5} M EDTA-Fe(III) instead of 10^{-4} M EDTA-Fe(III) indicated as CK2]; the low Fe treatment combined with foliar application of 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, or 10^{-6} M EBR, designated as EBR1, EBR2, EBR3, EBR4, and EBR5, respectively. The treatments were arranged in a randomized block design with three replicates, giving a total of 21 pots. The nutrient solution was adjusted to pH 6.3 and the EBR solutions were applied by foliar spray at a rate of 10 cm^3 per pot every morning (the controls sprayed with deionized water of equal amount). The plants were grown in a growth chamber at a relative humidity of 60 %, a 14-h photoperiod, an irradiance of 300 $\mu mol\ m^{-2}\ s^{-1}$, and day/night temperatures of 25/20 °C. The EBR was dissolved in a minimal volume of ethanol, and then brought into distilled water. After 21 d, the plants were harvested and the roots and shoots were separated and washed with 5 mM $CaCl_2$ first and then repeatedly with deionized water. Shoot height, root length, and fresh masses were measured. Root volume was determined by the water displacement method. For measurement of enzymes, fresh samples were frozen in liquid nitrogen and stored at -70 °C. For estimation of dry masses and concentrations of Fe and other minerals, plant samples were oven-dried at 105 °C for 30 min and then at 80 °C till the material reached its constant mass. About 0.2 g of dried leaves and roots were mineralized in 5 cm^3 of concentrated $HNO_3 + H_2SO_4 + HClO_4$ (5:1:1) supplemented with a few drops of H_2O_2. Content of Fe and other minerals in the digested solution was determined using atomic absorbance spectrometry (*Persee TAS-990*, Beijing, China) according to Graziano *et al.* (2002).

After 10 and 20 d of the treatments, Chl content was determined according to the method of Knudson *et al.* (1977). Fresh leaf tissue (0.5 g) was extracted with 2 cm^3 of 95 % (v/v) ethanol for 24 h in the dark, and the extract was analyzed for the amounts of Chl *a,* Chl *b*, and carotenoids (Car) by reading absorbances at 665, 649, and 470 nm using a spectrophotometer *Unicowf UV-2000* (China).

A membrane fraction enriched in plasma membrane vesicles was prepared as described by Briskin *et al.* (1987) with minor modifications. Excised roots were homogenized with a mortar and pestle in a cold grinding medium containing 25 mM HEPES-TRIS (pH 7.2), 250 mM mannitol, 5 mM Na_2-EDTA, 5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), and 1.5 % (m/v) polyvinyl pyrrolidone (PVP). All isolation procedures were carried out at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 560 *g* for 12 min, then the supernatant was centrifuged at 10 000 *g* for 15 min, and the supernatant was separated and centrifuged at 60 000 *g* for 30 min to yield a crude membrane fraction. The resultant pellet was resuspended in 1 cm^3 of a gradient buffer containing 20 mM HEPES-TRIS (pH 7.5), 5 mM Na_2-EDTA, and 0.5 mM EGTA. The supernatant was layered on the top of a step gradient consisting of 1 cm^3 of 45, 33, and 15 % (m/v) sucrose, respectively, and then centrifuged at 70 000 *g* for 2 h. The tonoplast-enriched fraction was collected at the 15/33 % sucrose interface and the plasma membrane (PM)-enriched fraction was collected at the 33/45 % sucrose interface. Each fraction was centrifuged at 100 000 *g* for 60 min. The resulting pellet was resuspended in a medium containing 20 mM HEPES-TRIS (pH 7.5), 3 mM $MgCl_2$, 0.5 mM EGTA, and 300 mM sucrose, then quickly frozen in liquid nitrogen and stored at -70 °C until used for enzyme assays.

Hydrolysis of ATP was performed as described by Briskin *et al.* (1987) in 0.5 cm^3 of a reaction medium containing 36 mM TRIS-MES (pH 6.5), 30 mM ATP-Na_2, 3 mM $MgSO_4$, 1 mM NaN_3, 50 mM KNO_3, 1 mM Na_2MoO_4, 0.02 % (v/v) *Triton X-100* in the presence or absence of 2.5 mM Na_3VO_4. The reaction was triggered by adding 50 mm^3 of the PM vesicles. After a 30 min incubation at 37 °C, the reaction was quenched by the addition of 50 mm^3 of 55 % (m/v) trichloroacetic acid. Activity of H^+-ATPase was determined by measuring the release of inorganic P (Ohinishi *et al.* 1975). Additionally, ATP hydrolysis was performed in 0.5 cm^3 of a reaction medium containing: 50 mM TRIS-ME (pH 7.6), 250 mM sucrose, 1.0 mM DTT, 2.0 mM ATP-Na_2, 0.1 mM $(NH_4)_2MoO_4$, 0.02 % (v/v) *Triton X-100*, 300 mM $NaNO_3$, 1.0 mM NaN_3 in the presence or absence of 2.0 mM $Ca(NO_3)_2$. The reaction was triggered by adding 5×10^{-3} cm^3 of the PM vesicles. After 30-min incubation at 37 °C, the reaction was quenched by the addition of 5×10^{-3} cm^3 of 55 % (m/v) trichloroacetic acid. Activity of Ca^{2+}-ATPase was determined by measuring the release of Pi (Ohinishi *et al.* 1975).

After the 21 d treatment, plant roots were immersed in a saturated $CaSO_4$ solution for 5 min, washed with deionized water, and then placed in a beaker with a nutrient solution which contained 0.1 mM Fe-EDTA and 0.4 mM 2,2'-bipyridyl, pH 5.0. The environmental conditions during the measurement were the same as for the plant growth. After 2 h, Fe-reduction capacity of roots was determined by measuring the concentration of the formed Fe^{2+}-bipyridyl complex at 532 nm using a spectrophotometer (Gao and Shi 2007).

After 10 and 21 d of the treatments, young and old leaves, stems, and roots were cut into pieces and extracted with 1 M HCl (tissue:HCl, 1:10, m/v) (Takker and Kaur 1984), shaken for 5 h and filtered, and the subsequent active Fe content in the filtrate was measured using an atomic absorption spectrophotometer (Gao and Shi 2007).

At harvest, roots were separated and immersed in 20 mM Na_2-EDTA for 15 min and then rinsed three times with deionized water to remove any Fe attached to the external surface of the roots. Fresh young leaves, old leaves, stem and roots (25 g) were homogenized in 50 cm^3 of a chilled extraction buffer containing 50 mM HEPES (pH 7.5), 500 mM sucrose, 1 mM DTT, 5 mM ascorbate, and 1 % PVP. The homogenate was centrifuged at 500 *g* for 5 min to isolate the cell wall fraction. The supernatant was then centrifuged at 20 000 *g* for 45 min to sediment cell organelles, and the resultant supernatant solution was referred to as the soluble fraction. All steps were performed at 4 °C. The fractions of the samples were digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, v/v) at 120 °C for at least 3 h (Su *et al.* 2014). The concentration of Fe was then determined using an atomic absorption spectrometer (Kong *et al.* 2014a).

For enzymatic analyses, samples were homogenized in a 50 mM phosphate buffer (K_2HPO4 + KH_2PO4; pH 7.8) using a mortar and pestle in liquid nitrogen. The homogenate was filtered through four layers of muslin cloth and centrifuged at 12 000 *g* and 4 ºC for 10 min. Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Stewart and Bewley (1980). One unite of SOD activity was defined as the amount of the enzyme required to cause a 50 % inhibition of the reduction of NBT as monitored at 560 nm. Catalase (CAT) activity was measured as a decline in absorbance at 240 nm due to consumption of H_2O_2 according to the method of Patra *et al.* (1978). Peroxidase (POD) activity was measured by an increase in absorbance at 470 nm due to guaiacol oxidation (Nickel and Cunningham 1969). One unit of POD was defined as an absorbance change of 0.01 per minute. Lipid peroxidation in fresh leaves was measured in terms of malondialdehyde (MDA) content by the thiobarbituric acid method (Heath and Packer 1968). Soluble protein content was determined according to Bradford (1976) with a Coomassie brilliant blue *G-250* reagent with bovine serum albumin as standard.

For measurement of $O_2^{\bullet-}$ generation rate, 0.3 g of fresh leaves or roots was homogenized in 3 cm^3 of ice cold 50 mM PBS (pH 7.0), and the homogenate was centrifuged at 10 000 *g* for 10 min. Then, 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of 50 mM PBS (pH 7.8) and 0.1 cm^3 of 10 mM hydroxylamine hydrochloride. The reaction mixture was incubated at 25 °C for 35 min. A 0.5 cm^3 of the above reaction mixture was then added to 0.5 cm^3 of 17 mM sulfanilamide and 0.5 cm^3 of 7.8 mM α-naphthylamine solution. After 20 min of reaction, 2 cm^3 of ether ($C_4H_{10}O$) was added into the above solution and mixed. The solution was centrifuged at 1 500 *g* and 4 °C for 5 min. The absorbance of the pink supernatant was measured at 530 nm. The absorbance values were calibrated to a standard curve generated with known concentrations of HNO_2 (Shi and Zhu 2008).

For determination of H_2O_2 content, 1 g of fresh leaves or roots was homogenized in 2 cm^3 of ice-cold acetone and centrifuged at 4 000 *g* for 15 min. A titanium reagent (2 %, v/v, $TiCl_2$ in conc. HCl) was added to the supernatant. A Ti-H_2O_2 complex together with unreacted Ti was then precipitated by adding 0.2 cm^3 of 17 M ammonia solution for each 1 cm^3 of extract, then centrifuged at 4 000 *g* for 15 min and after that, the supernatant was discarded. The precipitate was washed five times with ice -cold acetone by resuspension, drained in 1 M H_2SO_4 (3 cm^3). The absorbance of the solution was measured at 410 nm against blanks which had been similarly prepared but without plant tissue (Simaei *et al.* 2011).

Nitrate reductase (NR) activity was measured following the method of Jaworski (1971). Fresh leaf or root samples (0.5 g) were cut into small pieces and transferred to plastic vials containing 9 cm^3 of a phosphate buffer (K_2HPO_4 + KH_2PO_4, pH 7.5), potassium nitrate and isopropanol solution. The reaction mixture was incubated at 30 °C for 2 h, added 0.2 % (m/v) of N-1-naphthylethylenediamine dihydrochloride and 1 % (m/v) of sulfanilamide. Absorbance was read at 540 nm and compared with that of a calibration curve.

Free proline content in fresh leaf or root samples was determined by the method of Bates *et al.* (1973). The samples were extracted in sulfosalicylic acid. To the extract, equal volumes of glacial acetic acid and ninhydrin solution were added. The samples were heated at 100 °C, and 5 cm^3 of toluene was added. The absorbance of the superstratum layer was read at 528 nm.

Statistical analyses were carried out by analysis of variance (*ANOVA*) using the *SAS* software (*SAS Institute*, Cary, NC, USA). Differences between treatments were separated by the least significant difference test at a 0.05 probability level.

Results

The Fe deficiency (CK2) stress was reflected on shoot height and shoot and root masses as they were significantly reduced as compared to the CK1 (Table 1). However, the application of EBR improved plant growth and the best result was achieved at 10^{-7} M EBR (EBR3) which increased the shoot height, shoot mass, and root mass by 39.4, 66.9, and 17.9 %, respectively, in comparison with the CK2. However, the root length and deficient plants significantly decreased after 10 and 20 d of the treatment as compared to the CK1. The application

Table 1. Effects of different concentrations of 24-epibrassinolide (EBR, foliar spray) on shoot height, root length [cm plant^{-1}], root volume [cm^3 plant^{-1}], and shoot and root masses [g plant^{-1}] in peanut seedlings grown under Fe deficiency for 21 d. CK1 - control; CK2 - low Fe treatment; EBR1-5 - low Fe treatment combined with foliar application of 10^{-8} (EBR1), 5×10^{-8} (EBR2), 10^{-7} (EBR3), 5×10^{-7} (EBR4), and 10^{-6} (EBR5) M EBR. Means ± SDs, *n* = 3. Different letters after means within the same column indicate significant differences at $P < 0.05$.

Treatments	Shoot height	Root length	Root volume	Shoot mass	Root mass
CK1	17.80±1.47a	14.40±0.38ab	1.03±0.07abc	3.63±0.21a	0.37±0.03a
CK2	11.67±0.55e	15.40±1.11a	1.15±0.07a	2.39±0.19c	0.28±0.05b
EBR1	12.87±1.07de	14.83±0.42ab	1.02±0.09abc	2.60±0.36bc	0.27±0.02b
EBR2	13.70±0.62cd	15.07±0.70ab	1.06±0.05abc	2.97±0.04b	0.27±0.02b
EBR3	16.27±0.64ab	14.97±0.35ab	0.97±0.12bc	3.99±0.25a	0.32±0.03ab
EBR4	15.03±0.75bc	14.77±0.42ab	1.10±0.06ab	3.61±0.12a	0.32±0.02ab
EBR5	12.87±0.81de	14.12±0.49b	0.93±0.08c	2.79±0.27bc	0.29±0.01b

of EBR increased Chl content except the EBR1 and EBR5 treatments, and the EBR3 increased Chl *a*, Chl *b*, and Chl *a*+*b* by 54.1, 71.1, and 58.1 %, respectively, after 10 d (Fig. 1*A,C*) and by 16.9, 52.6, and 23.5 %, respectively, after 20 d (Fig. 1*B,D*). The Chl content in the EBR3 plants was even higher than in the CK1. However, the foliar application of EBR increased the content of Car at 20 d after the treatment (Fig. 1*F*).

The H^+-ATPase plays an important role in maintenance of intracellular and extracellular pH that influences Fe activity inside and outside a cell. Therefore, in this experiment, we determined activities of plasma membrane H^+-ATPase and Ca^{2+}-ATPase in leaves and roots. The results show that in the Fe deficient plants, the activity of H^+-ATPase was stimulated by 89.1 % in leaves and 22.1 % in roots compared with the CK1. In addition, the EBR2 and EBR3 treatments further increased the activity of H^+-ATPase by 48.7 and 93.4 %, respectively, compared with the CK2 in leaves (Fig. 2*A,B*). On the contrary, the Fe deficiency significantly inhibited the activities of Ca^{2+}-ATPase in leaves and roots. However, the foliar application of EBR markedly increased the activity of Ca^{2+}-ATPase, especially the EBR3 treatment which increased it by 150.5 % in leaves and 27.9 % in roots as compared to the CK2 (Fig. 2*C,D*).

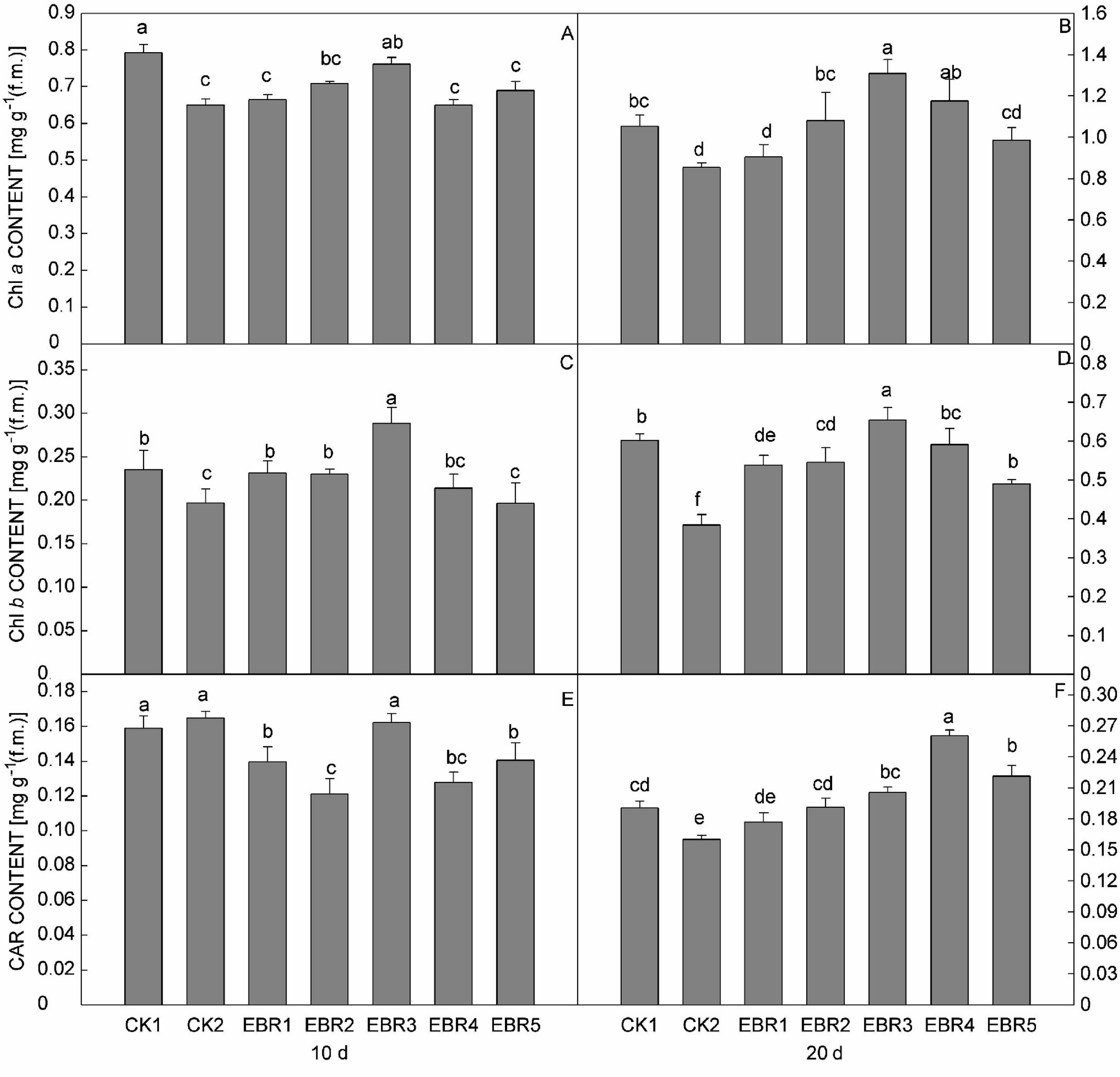

Fig. 1. Effects of different concentrations of 24-epibrassinolide (foliar spray) on chlorophyll (Chl) *a* (*A,B*), Chl *b* (*C,D*), and carotenoids (Car) (*E,F*) content in peanut seedlings grown under Fe deficiency for 10 or 20 d. Means ± SDs, *n* = 3. Different letters indicate significant differences at $P < 0.05$. Other details as in Table 1.

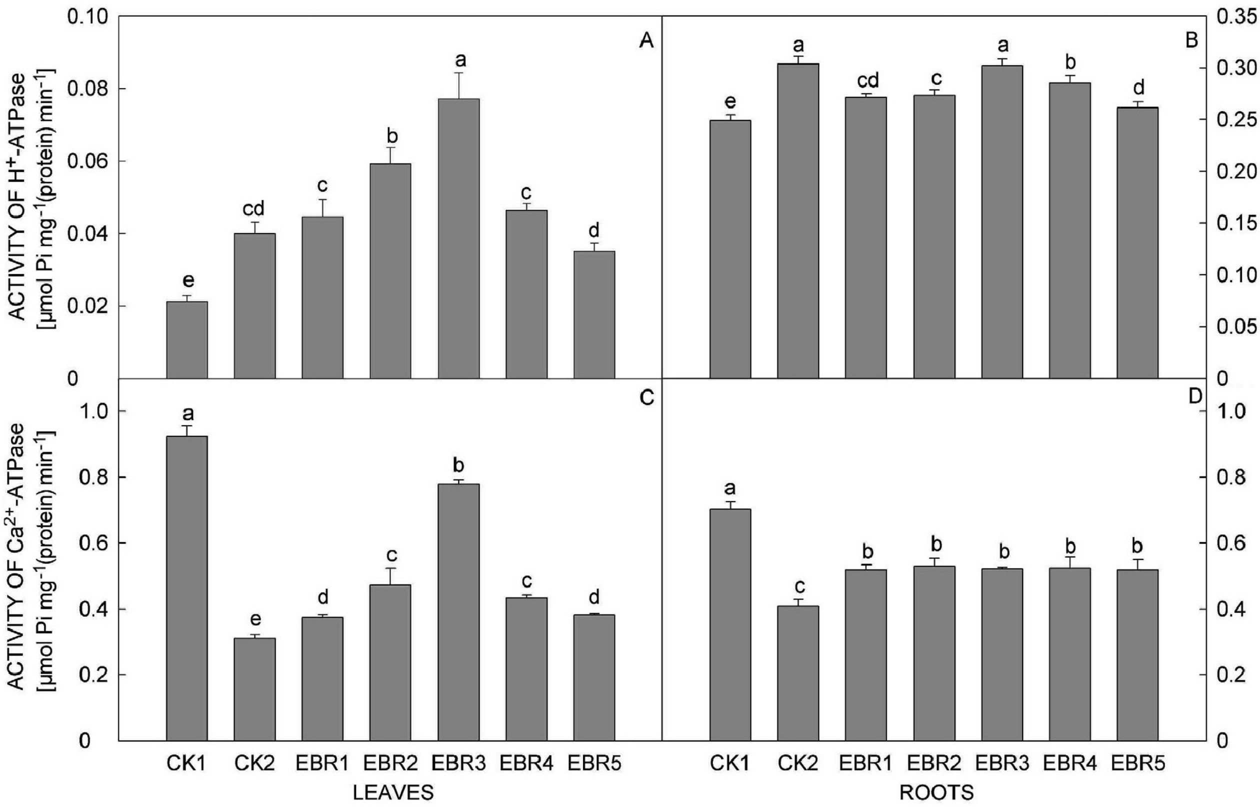

Fig. 2. Effects of different concentrations of 24-epibrassinolide (foliar spray) on activity of H^+-ATPase (*A,B*) and Ca^{2+}-ATPase (*C,D*), in leaves and roots of peanut seedlings with Fe deficiency for 7d. Means ± SDs, *n* = 3. Different letters indicate significant differences at $P < 0.05$. Other details as in Table 1.

Table 2. Effects of different concentrations of 24-epibrassinolide (foliar spray) on subcellular distribution of Fe [mg kg^{-1}(f.m.)] in young leaves, old leaves, stems, and roots in peanut seedlings grown under Fe deficiency for 21 d. Means ± SDs, *n* = 3. Different letters within the same row indicate significant differences at $P < 0.05$. Other details as in Table 1.

Fe content		CK1	CK2	EBR1	EBR2	EBR3	EBR4	EBR5
Young leaves	cell wall	52.07±0.81a	42.66±0.31b	41.31±0.62bc	40.58±1.00cd	36.60±1.74e	38.91±0.97d	42.70±0.89b
	cell organelles	24.02±0.24a	12.05±0.11d	12.86±1.22d	16.34±1.02c	21.05±1.73b	15.16±1.55c	12.39±0.25d
	soluble fraction	45.95±0.43a	32.93±0.92b	31.21±0.74c	29.13±0.50d	25.36±1.32e	28.27±1.39d	28.76±0.62d
Old leaves	cell wall	50.49±0.34a	34.00±2.00b	33.08±1.96bc	34.36±0.64b	30.98±2.52c	35.33±1.49b	33.28±1.15bc
	cell organelle	25.30±0.87a	17.67±0.95d	17.94±1.37d	18.17±0.45cd	20.30±0.50b	19.57±1.02bc	18.09±0.74cd
	soluble fraction	43.16±0.82a	32.67±0.55bc	33.99±0.85b	34.01±1.07b	31.05±1.05bc	32.43±1.28bc	33.45±0.88b
Stems	cell wall	43.22±1.26a	37.23±2.44b	31.84±0.93c	31.39±0.69c	27.11±1.48d	31.33±1.42c	31.54±1.16c
	cell organelle	27.89±1.41a	21.36±1.27b	22.44±0.54b	22.92±2.00b	22.80±1.07b	21.42±0.70b	22.24±1.07b
	soluble fraction	36.34±0.42a	23.45±1.08b	21.78±0.79c	23.86±0.17b	23.41±1.23b	19.76±0.73d	19.34±0.49d
Roots	cell wall	124.36±3.30a	69.14±1.89b	52.07±1.98d	51.96±1.65d	51.30±0.59d	54.11±0.92d	58.58±0.52c
	cell organelle	45.98±3.37a	27.29±1.11bc	25.65±0.71bc	25.99±1.46bc	28.52±0.52b	26.46±1.21bc	25.26±1.50c
	soluble fraction	45.98±3.37a	26.46±1.21b	17.98±0.48c	24.99±1.97b	24.19±0.68b	17.29±1.11c	18.60±0.64c

The Fe deficiency did not affect FCR activity. The EBR application increased FCR activity, and the EBR2 treatment resulted in the greatest increase in FCR activity, by 50.7 %, followed by the EBR3 and EBR4 with 29.8 and 29.7 %, respectively (Fig. 3). These results exhibit that the FCR activity in the plants was affected by external EBR.

The Fe deficiency significantly decreased active Fe content in plant tissues (young and old leaves, stems, and roots), especially after 21 d of the treatment. The foliar application of the different concentrations of EBR to the Fe deficient plants resulted in differential changes in plant active Fe content. In young leaves, the most effective concentration of EBR in increasing active Fe was 10^{-7} M (EBR3) which increased it by 82.3 and 64.7 % on day 10 and 21, respectively, after the treatment, compared to the CK2 (Fig. 4*A,B*), whereas in old leaves, the optimal concentration of EBR was 5×10^{-7} M (EBR4) which increased active Fe content by 28.8 and 59.2 % on day 10 and 21, respectively, as compared to the CK2 (Fig. 4*C,D*). The EBR2 treatment increased active Fe in stems and roots by 55.8 and 41.4 %, respectively, on day 10 after the treatment (Fig. 4*E,G*), whereas the EBR1 markedly increased active Fe content by 55.8 % in stems and the EBR4 by 41.4 % in roots on day 21 after the treatment, as compared to the CK2 (Fig. 4*F,H*). These results imply that EBR could improve Fe uptake and translocation in the Fe deficient plants.

The Fe deficiency significantly inhibited Fe uptake and translocation in the peanut seedlings resulting in a decrease in tissue Fe content by 17.6 % in young leaves, 12.6 % in old leaves, 24.0 % in stems, and 40.6 % in roots, as compared to the CK1 (Table 5). The foliar application of EBR increased total Fe content in young and old leaves but decreased it in roots with a minimal change in stems. In young leaves, the EBR3 and EBR4 treatments increased total Fe content by 16.0 and 13.8 %, respectively, similarly to the active Fe content (Table 5).

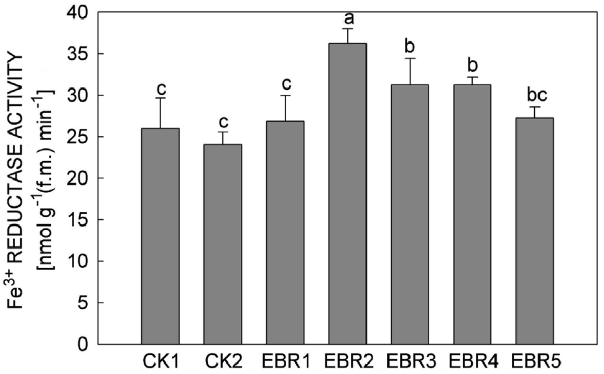

Fig. 3. Effects of different concentrations of 24-epibrassinolide (foliar spray) on Fe^{3+} reductase activity in roots of peanut seedlings under Fe deficiency for 21 d. Means SDs, $n = 3$. Different letters indicate significant differences at $P < 0.05$. Other details as in Table 1.

Table 3. Effects of different concentrations of 24epibrassiniolide on activities of superoxide dismutase (SOD) [U g^{-1}(f.m.)], peroxidase (POD) [U g^{-1}(f.m.) min^{-1}], catalase (CAT) [μmol H_2O_2 mg^{-1}(f.m.) min^{-1}], and nitrate reductase (NR) [μg g^{-1}(f.m.) min^{-1}] in leaves and roots of peanut seedlings grown under Fe deficiency for 21 d. Means ± SDs, $n = 3$. Different letters after means within the same column indicate significant differences at $P < 0.05$. Other details as in Table 1.

Organ	Treatments	SOD	POD	CAT	NR
Leaves	CK1	237.71±6.11e	22.71±1.80e	6.92±0.05a	0.36±0.009d
	CK2	263.20±5.09d	29.15±2.14d	6.48±0.55a	0.20±0.012e
	EBR1	274.79±12.23d	36.99±1.70c	6.65±0.34a	0.57±0.006b
	EBR2	311.80±2.97c	37.12±1.30c	6.65±0.17a	0.64±0.010a
	EBR3	312.12±7.80c	44.91±0.19b	7.02±0.51a	0.63±0.035a
	EBR4	337.57±6.43b	47.85±2.77b	6.94±0.24a	0.41±0.022c
	EBR5	432.16±9.20a	53.07±3.33a	6.92±0.05a	0.38±0.016d
Roots	CK1	190.71±8.18e	153.12±3.60a	1.90±0.06a	0.41±0.011c
	CK2	201.16±16.32de	73.46±4.45e	1.25±0.07c	0.24±0.011e
	EBR1	215.23±5.28cd	79.90±3.75cd	1.25±0.09c	0.37±0.009d
	EBR2	286.27±8.67a	86.61±3.33b	1.54±0.07b	0.60±0.011a
	EBR3	223.87±8.67bc	85.13±2.83bc	1.30±0.17c	0.47±0.008b
	EBR4	240.29±9.36b	79.22±3.88cb	1.61±0.07b	0.36±0.021d
	EBR5	224.94±5.18bc	76.66±3.34de	1.29±0.17c	0.43±0.020c

The Fe deficiency decreased Fe content in cell organelles by 49.8 % in young leaves, 30.2 % in old leaves, 23.4 % in stems, and 40.7 % in roots (Table 2). More Fe was found in cell organelles, cell wall, and soluble fraction in leaves after the foliar application of EBR to the Fe deficient plants. The EBR3 treatment attained the best results with an increase of Fe content in cell organelles by 74.7 % in young leaves and 14.9 % in old leaves. Meanwhile, the content of Fe in cell wall and soluble fraction was reduced by 14.2 and 23.0 %, respectively, in young leaves and 8.9 and 5.0 %, respectively, in old leaves. However, in stems and roots, the content of Fe did not change significantly in cell organelles although a marked decrease in Fe content occurred in cell wall after the foliar application of EBR to the Fe deficient plants.

In the Fe deficient plants, activity of SOD slightly increased in leaves and roots, but POD and CAT activities decreased by 52.0 and 34.5 %, respectively, in roots, as compared to the CK1. The foliar application of EBR to the Fe deficient plants stimulated the activities of the antioxidant enzymes (SOD, POD, and CAT) in leaves and roots (Table 3). The activities of SOD and POD in leaves increased with the increasing concentration of EBR applied. In roots, the EBR2 treatment enhanced the activities of SOD and POD more than any other treatment, by 42.3 and 17.8 %, respectively, whereas the EBR4 treatment was most effective in increasing CAT activity in roots (by 29.7 %). These results indicate that an optimal concentration of EBR was essential for stimulation of activities of the antioxidant enzymes.

The Fe deficiency increased MDA content by 94.9 % in leaves and 99.0 % in roots as compared to the CK1 (Table 4). However, there was no significant difference between the CK2 and CK1 with regard to rate of $O_2^{\bullet-}$ generation and H_2O_2 content in leaves although in roots,

Table 4. Effects of different concentrations of 24-epibrassinolide on superoxide anion ($O_2^{\bullet-}$) generation rate [μmol g^{-1}(f.m.) min^{-1}] and content of malondialdehyde (MDA) [nmol g^{-1}(f.m.)], H_2O_2 [μmol g^{-1} (f.m.)], soluble protein [mg g^{-1}(f.m.)], and free proline [μg g^{-1}(f.m.)] in leaves and roots of peanut seedlings grown under Fe deficiency for 21 d. Means ± SDs, *n* = 3. Different letters after means within the same column indicate significant differences at $P < 0.05$. Other details as in Table 1.

Organ	Treatments	$O_2^{\bullet-}$	MDA	H_2O_2	Soluble protein	Free proline
Leaves	CK1	0.061±0.005ab	17.64±2.33d	25.79±1.25ab	5.34±0.19c	12.79±0.69e
	CK2	0.067±0.007a	34.25±1.49a	26.93±0.70a	4.28±0.05d	17.18±0.99d
	EBR1	0.053±0.005ab	31.11±0.8b	25.69±0.97ab	7.76±0.17b	19.84±0.52c
	EBR2	0.058±0.014ab	33.29±1.36ab	25.00±0.86b	7.99±0.57ab	21.62±0.84b
	EBR3	0.056±0.005ab	24.14±1.57c	23.40±1.05c	8.39±0.27a	20.89±0.63bc
	EBR4	0.053±0.010b	24.75±1.90c	21.97±0.54c	8.27±0.23a	24.70±0.27a
	EBR5	0.059±0.008ab	26.39±1.53c	26.52±0.84ab	8.43±0.06a	24.64±0.63a
Roots	CK1	0.329±0.004f	30.23±0.60f	20.79±0.50b	4.40±0.24a	10.90±0.27b
	CK2	0.608±0.016a	60.05±1.20a	22.49±0.73a	3.89±0.35b	7.18±0.18d
	EBR1	0.422±0.023cd	49.06±2.13d	19.48±0.96c	4.33±0.07b	8.48±0.22c
	EBR2	0.410±0.014de	43.40±2.36e	12.92±0.72f	3.91±0.18b	8.63±0.11c
	EBR3	0.379±0.024e	29.29±2.00f	16.09±0.34e	3.64±0.17b	6.98±0.20d
	EBR4	0.442±0.026c	53.01±0.68c	17.30±0.52d	4.52±0.16a	11.50±0.19a
	EBR5	0.562±0.006b	56.49±2.27b	17.35±0.42d	3.56±0.14b	6.82±0.46d

Table 5. Effects of different concentrations of 24-epibrassinolide on concentrations of Fe, Ca, Mg [g kg^{-1}(d.m.)], Cu, and Zn [mg kg^{-1}(d.m.)] in young leaves, old leaves, stems and roots of peanut seedlings under Fe deficiency for 21 d. Means ± SDs, *n* = 3. Different letters after means within the same column indicate significant differences at $P < 0.05$. Other details as in Table 1.

Minerals	Organs	CK1	CK2	EBR1	EBR2	EBR3	EBR4	EBR5
Fe	roots	2.12±0.02a	1.26±0.02b	1.64±0.02c	1.08±0.01d	1.04±0.02e	1.08±0.02d	1.07±0.01de
	stems	0.48±0.02a	0.37±0.02bc	0.38±0.02bc	0.35±0.03c	0.40±0.03b	0.36±0.02bc	0.46±0.03a
	young leaves	0.64±0.03a	0.55±0.03c	0.62±0.02b	0.64±0.07ab	0.75±0.03ab	0.73±0.01ab	0.55±0.01b
	old leaves	0.76±0.03b	0.66±0.04c	0.71±0.04b	0.72±0.02b	0.72±0.01a	0.74±0.02a	0.71±0.01c
Ca	roots	0.57±0.12a	0.27±0.03c	0.27±0.02c	0.28±0.01c	0.43±0.11b	0.51±0.003ab	0.31±0.03c
	stems	2.40±0.19b	2.36±0.34b	2.95±0.84ab	2.56±0.33ab	2.51±0.27ab	3.02±0.26ab	3.20±0.41a
	young leaves	5.05±0.45cd	4.23±0.12d	6.44±0.72b	4.47±0.60d	5.65±0.37bc	6.46±0.15b	7.31±0.55a
	old leaves	11.42±0.65a	9.00±0.19c	1.10±0.06ab	9.41±1.33bc	11.44±0.59a	9.52±0.99bc	7.79±1.30c
Mg	roots	1.12±0.14ab	0.74±0.05d	1.13±0.10a	0.94±0.11bc	0.99±0.05abc	1.15±0.15a	0.85±0.09cd
	stems	0.40±0.05a	0.45±0.02a	0.47±0.12a	0.49±0.07a	0.46±0.05a	0.47±0.06a	0.48±0.06a
	young leaves	0.49±0.01c	0.39±0.10d	0.49±0.08c	0.53±0.04bc	0.57±0.03bc	0.63±0.04ab	0.67±0.02a
	old leaves	0.94±0.06a	0.52±0.04b	0.60±0.06b	0.66±0.03b	0.89±0.24a	0.92±0.10a	0.52±0.07b
Cu	roots	8.33±0.05b	10.13±0.51a	8.06±0.64b	0.99±0.07a	10.98±0.78a	10.63±0.82a	8.09±0.47b
	stems	1.24±0.13c	1.75±0.09a	1.43±0.07b	1.27±0.14bc	1.30±0.06bc	1.41±0.07b	1.30±0.09bc
	young leaves	1.45±0.17d	2.97±0.16a	1.81±0.17c	1.83±0.08c	1.88±0.09bc	2.11±0.18b	1.87±0.07bc
	old leaves	2.08±0.08c	3.80±0.11a	2.18±0.33c	2.36±0.13c	2.94±0.19b	3.28±0.19b	2.42±0.32c
Zn	roots	30.26±1.22ab	28.70±1.71bc	27.17±1.84c	28.75±2.07bc	31.07±1.40ab	31.66±0.70a	23.30±1.92 d
	stems	13.58±1.35bc	11.58±1.21d	14.56±0.50b	14.85±0.45b	12.50±0.79cd	14.38±0.24b	17.94±0.90 a
	young leaves	21.49±0.61b	18.41±1.82c	16.07±0.87d	21.26±0.17b	21.28±1.52b	23.00±1.87ab	24.73±0.34a
	old leaves	25.34±2.41a	21.59±1.89b	21.26±2.12b	21.89±2.31b	22.98±0.03ab	25.22±1.56a	17.43±1.43c

$O_2^{\bullet-}$ generation rate of the CK2 significantly increased by 84.7 %. The foliar application of EBR to the Fe deficient plants markedly reduced MDA content in leaves and roots with the EBR3 treatment being most effective, which decreased MDA content by 29.7 and 51.2 %, respectively, in leaves and roots. Rate of $O_2^{\bullet-}$ generation and H_2O_2 content decreased after the EBR application as well, but the change was different between leaves and roots. The EBR4 treatment was most effective in reducing $O_2^{\bullet-}$generation rate and H_2O_2 content in

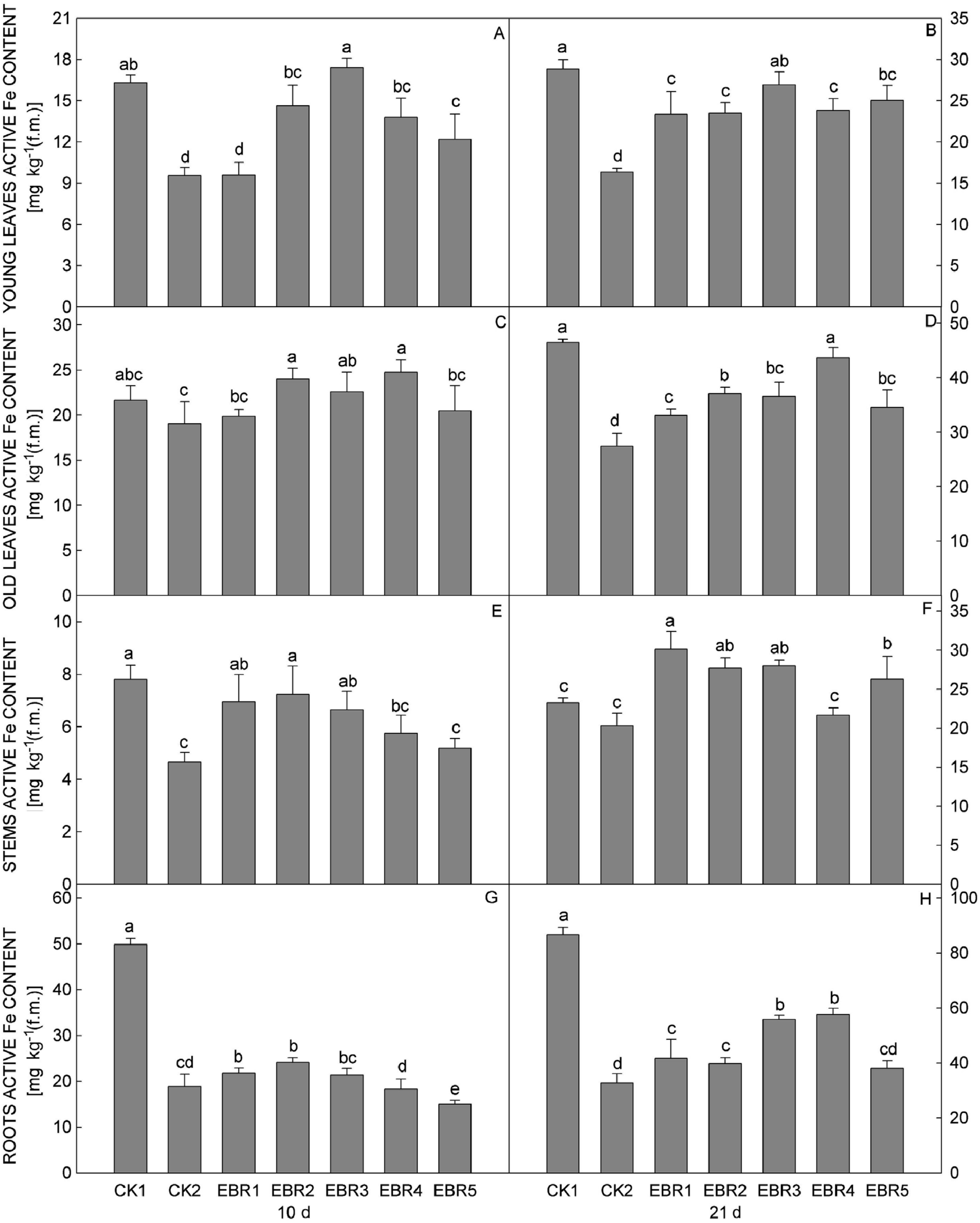

Fig. 4. Effects of different concentrations of 24-epibrassinolide on active Fe content in young leaves (*A,B*), old leaves (*C,D*), stems (*E,F*), and roots (*G,H*) of peanut seedlings under Fe deficiency for 10 and 21 d. Means ± SDs, $n = 3$. Different letters indicate significant differences at $P < 0.05$. Other details as in Table 1.

leaves, but the EBR3 treatment decreased more $O_2^{\bullet -}$ generation rate in roots and the EBR2 treatment was more effective in reducing H_2O_2 content in roots (Table 4).

The Fe deficiency reduced activity of NR and soluble protein content by 45.0 and 19.8 %, respectively, in leaves, and 41.2 and 11.6 %, respectively, in roots (Tables 3 and 4). The decreases in activities of NR and soluble protein content were partially reversed by the foliar application of EBR. Activities of NR varied with the different concentrations of EBR applied, but soluble protein content was little affected by EBR concentration. A maximum activity of NR was recorded in leaves and roots at the EBR2 treatment.

Free proline content in leaves remarkably increased in the Fe deficient plants, and the EBR4 treatment further increased content of free proline in leaves by 43.7 % compared with the CK2. However, only the EBR4 treatment attained a significant increase of free proline content in roots as compared to the CK1 (Table 4).

The Fe deficiency dramatically disturbed ion homeostasis in different parts of the plant; it significantly reduced content of Ca, Mg, and Zn by 52.6, 33.9, and 5.2 % in roots, and 21.1, 44.6, and 14.8 % in old leaves, respectively. However, concentration of Cu remarkably increased in roots and young leaves as compared to the CK1. The foliar application of EBR alleviated this imbalance, especially in roots, young leaves, and old leaves. Content of Ca significantly increased by the EBR3 treatment, and that of Mg and Zn markedly increased by the EBR4 treatment, whereas content of Cu decreased as compared to the CK2. However, mineral element content in stems did not change by the treatments except for Cu, which significantly increased under the Fe deficiency and slightly decreased by the EBR application as compared to the CK2 (Table 5).

Discussion

Brassinosteroids are ubiquitous in the plant kingdom (Bajguz and Tretyn 2003) and belong to a new group of plant hormones with significant growth-promoting activities (Bartwal *et al.* 2013). In this study, the application of EBR at concentrations of 10^{-8} - 10^{-6} M to the Fe deficient plants improved growth of the peanut seedlings, and 10^{-7} M EBR appears to be an optimal concentration (Table 1). This may be attributed to the fact that EBR can regulate cell division and elongation by activating cell wall loosening enzymes (Khripach *et al.* 2000). Cerana *et al.* (1983) have demonstrated that H^+-ATPase can activate the cell wall loosening enzymes by acidifying the apoplast. The role of EBR in enhancing the growth of the seedlings under Fe deficiency might be due to the activation of H^+-ATPase (Fig. 2). Arora *et al.* (2008) reported the positive effect of 10^{-9} M 28-homobrassinolide in a growth medium on shoot length of maize under salinity stress. Similarly, it has been reported that under Ni stress, application of 24-EBR increases shoot length and number of leaves in *Brassica juncea* (Kanwar *et al.* 2013). The content of Chls has been used as indicator of Fe status in plants (Graziano *et al.* 2002), as their common precursor is aminolevulinic acid (ALA), and the rate of ALA formation is controlled by Fe (Pushnik and Miller 1989). Besides, Fe atoms are directly involved in the electron transport chain and act as essential component in enzymatic reactions in photosynthesis (Marschner 1995). Under Fe deficiency, plant leaves generally have low photosynthetic activity due to several reasons. Firstly, carotenoids, and more specifically lutein and the xanthophylls (violaxanthin + antheraxanthin + zeaxanthin) are less affected than chlorophylls, and these pigment changes are associated to changes in leaf absorbance and reflectance. Therefore, in chlorotic leaves the amount of light absorbed per Chl unit increases but decreases in the actual photosystem (PS) II efficiency at steady-state photosynthesis, due to decreases in photochemical quenching and intrinsic PS II efficiency (Abadía *et al.* 1999), which results in a high risk for photoinhibition and photooxidative damages in leaves. Secondly, a low content of Chls impairs photosynthetic electron transport. In this study, the pigments content significantly increased (Fig. 1), as EBR could protect Chls from degradation in the Fe deficient plants by increasing the amount of the central component of Chl molecules - Mg (Table 5) and the content of active Fe in leaves (Fig. 4). As the Chl content increased, the photosynthetic rate was enhanced, thus producing more assimilates and subsequently promoting plant growth and development. Previous studies has reported that EBR enhance plant tolerance to various environmental stresses by improving photosynthesis (Ogweno *et al.* 2008, Piñol and Simón 2009), and EBR significantly increases fresh masses of shoots and roots and Chl content in mung bean under Al stress (Ali *et al.* 2008).

During evolution, Fe deficiency enhanced FCR activity and stimulated the plasma membrane proton pump, thus increasing proton exudation (Vert *et al.* 2002, Jin *et al.* 2006). The importance of FCR activity in development of Fe deficiency tolerance was demonstrated by over-expression of a yeast FCR gene in rice. Previous results revealed that transgenic plants with elevated FCR activity show a higher tolerance to Fe deficiency compared to non-transgenic plants (Ishimaru *et al.* 2006). However, in this study, the activity of FCR decreased in the Fe deficient plants as compared to the Fe sufficient plants (Fig. 3). We measured the FCR activity on day 21 and as time went on the activity of FCR decreased in the Fe deficient plants (Bacaicoa *et al.* 2009). As the FCR activity decreased, it was hard for the

Fe (III) to be reduced to Fe (II) in plant cells. The FCR activity increased by the foliar application of EBR to the Fe deficient plants, thus enhancing Fe (III) reduction to Fe (II) and consequently increasing the content of active Fe in the plants (Fig. 4). However, Wang *et al.* (2012) demonstrated that EBR likely plays a negative role in regulating Fe deficiency induced FCR activity as well as Fe translocation from roots to shoots in *Cucumis sativus*. The discrepancy may be due to a difference in the application method of EBR and plant species. Frédéric *et al.* (2007) has reported that H^+-ATPase also plays an important role in increasing active Fe content and transport of protons out of a cell across the plasma membrane, thus establishing a proton electrochemical gradient that contributes to the maintenance of intracellular and extracellular pH and an increased FCR activity. In this study, the activity of H^+-ATPase significantly increased in leaves and roots after the 7 d treatment under the Fe deficiency. The foliar application of EBR to the Fe deficient plants increased the H^+-ATPase activity in leaves but decreased it in roots (Fig. 2). Under the Fe deficiency, shoots were subjected to Fe starvation which significantly stimulated the activity of H^+-ATPase in roots to absorb more Fe and translocate it to shoots. On the other hand, EBR increased the H^+-ATPase activity and active Fe content in leaves (Fig. 4). Thus, the content of active Fe correlated with the H^+-ATPase activity when EBR was applied to the Fe deficient plants. However, total Fe content significantly increased only in leaves (Table 5). The results also show that the content of active Fe increased in leaves during the experiment and the active Fe was translocated from old leaves to young leaves after the EBR application. Active Fe was identified as better nutritional Fe indicator than total Fe (Hakan and Vahap 2007) as active Fe represents the portion of the total Fe that is readily assimilated by a plant. In order to understand the mechanisms of available Fe increase in leaves, we determined Fe distribution in subcellular structures of young leaves, old leaves, stems, and roots. The results show that the content of Fe in cell organelles significantly increased by the EBR application. In addition, vacuoles are the main cellular storage compartment where Fe is taken up by the vacuolar iron transporter 1 (Kim *et al.* 2006). When plants are subjected to Fe starvation, Fe may be released from the vacuoles. Extensive studies have been conducted on the mechanisms of Fe transport between the apoplast and symplast and between the cytoplasm and organelles (Thomine *et al.* 2003); the aspects of plant Fe mobilization by EBR were not well understood.

Iron deficiency inhibits antioxidant systems and increases production of ROS, that impairs the electron transport chains in chloroplasts and mitochondria (Graziano *et al.* 2005), thus resulting in oxidative stress. In this study, the activities of POD and CAT were significantly inhibited, and SOD slightly increased under the Fe deficiency (Table 3). It is well-known that SOD can detoxify $O_2^{\bullet-}$ in different cell organelles by formation of H_2O_2 (Mittler *et al.* 2002) and may contain Cu, Zn, Mn, or Fe as metal components (Sevilla *et al.* 1984). Under Fe deficiency, Fe-SOD activity is low (Iturbe-Ormaetxe 1995), but CuZn-SOD activity increases, which results in an increased total SOD activity and H_2O_2 production (Tewari *et al.* 2005). The increased ROS production led to the accumulation of MDA (Table 4), an indicator of oxidative damage to membranes, and it increases plasma membrane leakage (Hajlaoui *et al.* 2009), damages to DNA, lipids, proteins, and Chls (Jaleel *et al.* 2009) and disrupts redox homeostasis (Gille and Sigler 1995). The foliar application of EBR to the Fe deficient plants enhanced the activities of antioxidant enzymes (SOD, POD, and CAT) and soluble protein content, reduced the ROS production and MDA content, and thereby protected membranes from lipid peroxidation and oxidative stress. Kanwar *et al.* (2013) reported that treatment with 24-EBR significantly modulates activities of SOD, POD, and CAT and protein content under Ni stress. Similarly, application of BRs increases accumulation of proline and enhances activities of antioxidant enzymes in salt stressed *Cicer arietinum* (Ali *et al.* 2007) and *Vigna radiata* (Hayat *et al.* 2010). These results indicate that EBR can modulate the activities of the enzymes and mitigate adverse effects of Fe deficiency.

Activity of NR, a key enzyme in nitrogen assimilation, measures the ability of nitrate utilization in plants (Larcher 1995). In this study, the Fe deficiency significantly decreased the activity of NR, which agrees with previous results. Alcaraz *et al.* (1986) reported that activity of NR is lower in leaves of Fe deficient plants, but can be restored by resupplying Fe. However, foliar application of EBR to Fe deficient plants elevates activity of NR, thus affecting synthesis of proteins or enzymes (Sasse 2003). Ali *et al.* (2008) reported that under Al stress, application of BRs improves activity of NR in *Vigna radiata*. Besides, proline acts as osmoprotectant (Hartzendorf and Rolletschek 2001), membrane stabilizer (Bandurska 2001), and ROS scavenger (Matysik *et al.* 2002) under extreme conditions. However, the soluble protein and free proline content decreased under the Fe deficiency (Table 4), as Fe is required for protein synthesis especially in chloroplasts. On the other hand, protein content is up-regulated in leaves of plants treated with 24-EBR alone (Arora *et al.* 2012) and proline content in BR treated mung bean hypocotyl segments under stress conditions (Zhao and Chen 2003). Also, Sirhindi *et al.* (2009) reported that EBR treatment increases protein content of seedlings of B. *juncea* under natural field conditions, and Fariduddin *et al.* (2013) evidenced that application of EBR increases content of proline in association with NaCl and Cu stresses.

The beneficial effect of EBR on plant growth could also be attributed to the maintenance of optimal mineral nutrition. In the peanut plants, the Fe deficiency dramatically disturbed Ca, Mg, Cu, and Zn homeostasis. However, the EBR application promoted uptake and translocation of these mineral elements (Table 5). The application of EBR enhanced the activities of H^+-ATPase and Ca^{2+}-ATPase in roots and leaves (Fig. 2). H^+-ATPase can establish an electrochemical potential gradient to maintain ion balance in plants (Palmgren 2001), and Ca^{2+} acts as intracellular messenger in coupling a wide range of extracellular signals to specific responses (Alex *et al.* 1996) and so increases ion uptake and translocation. With the increased content of Ca, Mg, and Zn, the photosynthesis and the activities of enzymes were improved (Table 3) as Mg plays an essential role in photosynthesis (Marschner 1995) and holds a critical role in activation of ATPase, ribulose-1,5-bisphosphate carboxylase/oxygenase, and protein kinases (Cakmak and Kirkby 2008), and Cu and Zn are essential in other enzymatic reactions. Bajguz (2000) reported that 10^{-8} M EBR decreases accumulation of Cu, Cd, and Zn in cells of *Chlorella vulgaris*. Kanwar *et al.* (2013) evidenced that BRs confer tolerance against excess of Ni, Cu, and Mn in *B. juncea* by reducing their uptake and stimulating antioxidant enzymes. To fully understand the mechanisms of EBR in regulating ion balance in plants under Fe deficiency stress, further research is needed.

In conclusion, application of EBR at a proper concentration could alleviate the Fe deficiency symptom in peanut. The potential mechanisms include: *1*) increased content of Chls, soluble proteins, and free proline; *2*) regulated activities of key antioxidant enzymes to eliminate ROS; *3*) enhanced H^+-ATPase and Ca^{2+}-ATPase activities causing mineral element uptake and translocation; and *4*) increased Fe uptake and translocation from roots to shoots. Therefore, the EBR application could improve the growth and development of the plants under the Fe deficiency, and the optimal concentration appears to be 10^{-7} M.

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