INFLUENCE OF CALCIUM DEFICIENCY ON SUPEROXIDE GENERATING-NADPH OXIDASE AND ANTIOXIDANT DEFENSE SYSTEMS IN BEAN ROOT CELLS

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ABSTRACT

Calcium (Ca) is an essential mineral nutrient element involved in growth and development processes of plants. There are several physiological processes, which are greatly affected by Ca nutritional status of plants such as cell extension, cell wall stabilization, signal transduction and membrane transport. Based on the studies in mammalian systems Ca was found to be effective on activity of superoxide radical ($O_2^{\cdot -}$) generating NADPH oxidases and antioxidative defense systems. The effects of Ca nutritional status of plants on NADPH oxidase and antioxidative defense systems in plant cells are rarely studied. In the present MSc study, the role of Ca on $O_2^{\cdot -}$ generating NADPH oxidase and antioxidative defense systems (i.e., superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase, ascorbic acid and non-protein sulfhydryl-compounds) was studied in cytosolic fractions of root cells by using bean (*Phaseolus vulgaris*, cv. Nassua) plants grown in nutrient solution with different Ca supplies (50 μM to 2000 μM).

The results obtained show that exposure of Ca-adequate plants (2000 μM Ca supply) to different levels of deficient Ca supply (50 μM to 250 μM Ca) reduced the dry matter production and elongation of roots and caused brownish color formation along the roots. The decrease in root growth caused by Ca deficiency was more severe than the decrease in shoot growth. Compared to Ca-sufficient plants, the levels of NADPH oxidase and NADPH-dependent $O_2^{\cdot -}$ generation were distinctly decreased in Ca-deficient plants. This decrease was more pronounced at the lowest Ca supply (50 μM). Resupply of Ca to Ca-deficient roots for 8, 24 and 48 h markedly enhanced the activity of NADPH oxidase and the NADPH-dependent $O_2^{\cdot -}$ generation. Pretreatment with the inhibitor of NADPH oxidase, diphenylene iodonium (DPI), inhibited the rate of NADPH-dependent $O_2^{\cdot -}$ generation by around 50 %. Analysis of antioxidant defense

systems showed that Ca deficiency did not effect the ascorbic acid concentration, but markedly reduced the concentration of non-protein sulfhydryl (SH)-compounds (predominantly glutathione). Of the antioxidant enzymes, the superoxide dismutase, ascorbate peroxidase and catalase were not affected by Ca deficiency stress in roots. However, the activity of glutathione reductase was severely diminished by Ca deficiency, indicating a particular affect of Ca on redox status of glutathione in roots. The results indicate that Ca is required for generation of $O_2^{\cdot -}$ by activating NADPH oxidase in root cells. As NADPH-dependent $O_2^{\cdot -}$ generation is involved in adaptive response of plants to different biotic and abiotic stress factors, maintenance of high activity of NADPH oxidase by adequate Ca supply was considered as a fundamental role of Ca in growth and development processes of plants.

Keywords: calcium deficiency, *Phaseolus vulgaris*, root, NADPH oxidase, superoxide radical, antioxidant defense system

ÖZET

Kalsiyum (Ca) bitkilerin büyüme ve gelişmesi için mutlak gerekli bir besin elementidir. Örneğin hücre genişlemesi, hücre duvarının güçlenmesi, sinyal iletimi ve membran taşınımı gibi birçok fizyolojik süreç bitkilerin Ca beslenmesinden çok etkilenmektedir. Memeli sistemlerinde yapılan çalışmalara göre, superoksit radikal üreten NADPH oksidaz aktivitesi ve antioksidatif savunma sistemleri üzerinde Ca'un önemli etkilisi bulunmaktadır. Bitkideki Ca'un NADPH oksidaz ve antioksidatif savunma sistemleri üzerindeki etkisi çok az çalışılmıştır. Bu yüksek lisans çalışmasında Ca'un süperoksit üreten NADPH oksidaz ve antioksidatif savunma sistemleri (süperoksit dismutaz, askorbat peroksidaz, glutation redüktaz, katalaz, askorbik asit ve SH-grupları) üzerindeki rolü değişik oranlarda Ca içeren (50 μM'dan 2000μM'a) besin çözeltilerinde yetişen fasulye bitkisinin (*Phaseolus vulgaris,* cv Nassua) kök hücrelerinin sitozolik fraksiyonu kullanılarak çalışılmıştır.

Elde edilen bulgular, yeterli miktarda Ca (2000 μM) ile beslenen bitkilere farklı oranda düşük Ca dozları (50 μM'dan 2000μM'a) uygulandığında kuru madde üretiminin ve köklerin uzamasının azaldığını ve köklerin kahverengileştiğini göstermiştir. Kalsiyum eksikliğinin neden olduğu kök büyümesindeki azalmanın yeşil aksam büyümesindeki azalmaya oranla daha şiddetli olduğu bulunmuştur. Yeterli Ca ile beslenen bitkilerle karşılaştırıldığında NADPH oksidaz aktivitesi ve NADPH'a bağımlı $O_2^{\cdot -}$ üretimi Ca noksanlığındaki bitkilere ölçütle azalmıştır. Bu azalma en düşük Ca uygulamasında daha belirgin olmuştur. Kalsiyum eksikliğindeki köklere 8, 24 ve 48 saat süreyle Ca uygulaması NADPH oksidaz aktivitesini ve NADPH'a bağımlı $O_2^{\cdot -}$ seviyesini tekrar arttırdığını göstermiştir. NADPH oksidazın inhibitörü, dipenil iyodiniyum (DPI), NADPH'a bağımlı $O_2^{\cdot -}$ üretiminin % 50 oranında azalmasını sağlamıştır. Antioksidatif savunma sistemlerinin analizlerinde Ca eksikliği askorbik

asit konsantrasyonunu etkilemezken, SH-gruplarının (temel glutation) konsantrasyonunu ise belirgin bir biçimde azaltmıştır. Antioksidatif enzimlerden, süperoksit dismutaz, askorbat peroksidaz ve katalaz aktivitesi köklerde Ca eksikliği stresinden etkilenmemiştir. Ancak, glutation redüktaz aktivitesinin Ca eksikliğinden dolayı büyük oranda azalması, köklerdeki glutationun redox durumu üzerinde Ca'un etkisinin olduğunu göstermektedir. Sonuçlar, kök hücrelerinde NADPH oksidaz aktivasyonuyla oluşan $O_2^{\cdot-}$ üretimi için Ca'un gerekli olduğunu işaret etmektedir. NADPH'a bağımlı $O_2^{\cdot-}$ üretimi bitkilerin değişik biyotik ve abiyotik stres faktörlerine karşı adaptasyonunda rol almasından dolayı Ca'un NADPH oksidaz üzerindeki bu olumlu etkisi, onun bitkilerin çevresel etmenlere karşı adaptasyonda ne denli önemli olduğunu göstermektedir.

Anahtar Kelimeler: kalsiyum eksikliği, *Phaseolus vulgaris*, kök, NADPH oksidaz, süperoksit radikali, antioksidatif savunma sistemi

To my family

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ABBREVIATIONS

AP: Ascorbate peroxidase

AsA: Reduced ascorbic acid

DHAsA: Oxidized ascorbic acid

Ca: Calcium

CAT: Catalase

DHAsA: Dehydroascorbate

DW: Dry weight

FW : Fresh weight

GR: Glutathione reductase

GSH: Reduced glutathione

GSSG: Oxidized glutathione

$OH^{\cdot}$: Hydroxyl radical

H_2O_2 : Hydrogen peroxide

µg: microgram

µM: micromole

mg: milligram

MDAsA: Monodehydroascorbate

NADPH : Nicotinamide adenine dinucleotide

ROS: Reactive oxygen species

$O_2^{\cdot -}$:Superoxide radical

$^1O_2$: Singlet oxygen

SOD: Superoxide dismutase

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1 INTRODUCTION

High productivity and healthy growth of plants rely on adequate and balanced supply of mineral nutrient elements. Limited supply of one of these mineral nutrients adversely affects the productivity of plants. Calcium (Ca) is one of the major essential nutrient element that has an effect on both plant growth, development and also quality of harvested plant parts (Marschner, 1995; Mengel and Kirkby, 2001; Rudd and Franklin-Tag, 2001; Foreman et al., 2003). Plants suffer from Ca deficiency stress because of the factors that prevent either the availability of Ca in the soil (soil acidity, Al toxicity) or limit the transport of Ca into the actively growing points of plants; i.e., sink organs, young leaves, fruits, shoot apex (i.e., high humidity). Major reasons for limited Ca transport into the growing points of plants are extremely low mobility of Ca in phloem and very low transpiration capacity of the sink organs (Marschner, 1995; Golez and Kyuma, 1997; Mengel and Kirkby, 2001; Fageria, 2002; Goenaga and Smith, 2002). Well-known disorders of Ca deficiency in plants include blossom-end rot in tomatoes and peppers, tipburn in lettuce, and bitter pit in apples (Saure, 1998, 2001; Mengel and Kirkby, 2001; Schmitz-Eiberger et al., 2002).

In recent years, Ca has attracted much interest in plant physiology and molecular biology because of its function as a second messenger in the signal conduction between environmental factors and plant responses in terms of growth and development (Price et al., 1994; Sanders et al., 1999; Rudd and Franklin-Tag, 2001). When taken up into the plant system, Ca is involved in the regulation of plant responses to various biotic and abiotic stresses by contributing either directly or indirectly in plant defense mechanisms

During biotic stress conditions (e.g., elicitor or pathogen attack), Ca plays a pivotal role in regulation of reactive oxygen species (ROS) production, primarily

superoxide ($O_2^{\cdot-}$) and H_2O_2, by activating of $O_2^{\cdot-}$ generating NADPH oxidase. Activation of $O_2^{\cdot-}$-generating NADPH oxidase leads an oxidative burst (stress) at the site of pathogen attack (Sagi and Fluhr, 2001). Also, Ca serves as a common component of signal transduction pathways induced by many different stomatal closing signals during pathogen attack and also during abiotic stress as shown at heat stress (McAinsh et al., 1996; Lee et al., 1999; Rudd and Franklin-Tag, 2001; Fu and Huang, 2003).

It is well documented that ROS are highly toxic to plant cells and pathogens and can involve in signal transduction (Mendy et al., 1996; Grant and Loake, 2000; Rudd and Franklin-Tog, 2001). Plant cells are well equipped against damaging attack of ROS and possess antioxidative defense mechanisms to detoxify ROS, such as superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT) (Mittler, 2002.).

Besides the contribution to the pathogen-induced oxidative burst in plants (Grant et al., 2000; Rudd and Franklin-Tog, 2001), Ca also alleviates the oxidative damage caused during abiotic stresses (heat, water, and drought) by affecting the activity of antioxidants (GR, SOD and CAT) and decreasing the membrane lipid peroxidation (Jiang and Huang, 2001; Nayyar and Kaushal, 2002a, 2002b; Fu and Huang, 2003).

Although there are a large number of studies dealing with the requirement of Ca in either plant growth and development or responses to biotic and abiotic stresses, limited research has been conducted concerning effects of Ca supply on $O_2^{\cdot-}$ generating NADPH oxidase activity and the antioxidative defense system in plant cells (Schmitz-Eiberger et al., 2002). Therefore, in this MSc thesis project, the effects of varied supply of Ca on the activity of $O_2^{\cdot-}$ generating NADPH oxidase and antioxidative defense system in bean roots were investigated. Experiments were conducted under controlled environmental conditions using bean plants (cultivar Nassua) by measuring the activity of $O_2^{\cdot-}$ generating NADPH oxidase and the level of antioxidative defense system in cytosolic fractions of root cells. Additionally, root and shoot dry matter production and Ca concentrations were measured at different Ca supplies.

2 PREVIOUS STUDIES

2.1 Calcium in Soil and Plant

The mean Ca concentration on earth's crust amounts to about 36.4 g/kg. Although its concentration is high in soil, there are several plant and climatic factors that either influence the availability of Ca in soil or restrict the transport of Ca inside the plant cells. The most common one that influences the availability of Ca in soils is soil acidity (Golez and Kyuma, 1997; Fageria, 2002; Goenaga and Smith, 2002). Acid soils usually contain very high concentration of toxic Al, which limits chemical availability and uptake of Ca, and enhances precipitation of Ca in soils and blocking Ca channels in the plasma membrane, and thus causing occurrence of Ca deficiency in plants (Marschner, 1995; Mengel and Kirkby, 2001; Fageria, 2002; Goenaga and Smith, 2002). Interestingly, under certain conditions plants can suffer from Ca deficiency stress despite huge amounts of plant available Ca in soils and high Ca concentration in plants. This is mainly because; the absorbance of Ca by roots or the translocation of Ca to the sink organs is prevented by either high humidity or the salt concentration of growth medium (Choi et al., 1997).

Mineral nutrition is an important external factor involved in plant growth and yield formation. For the production of fruits and vegetables with high quality, an adequate Ca supply is particularly important. If the xylem sap is low in Ca or the rate of transpiration of the fruits is poor, as occurs under humid conditions, inadequate levels of Ca can move into the fruits resulting in formation of Ca deficiency symptoms. In tomato Ca deficiency disease is known as "blossom-end rot" and is characterized by a

cellular breakdown at the distal end of the fruit (Saure, 2001; Schmitz-Eiberger et al., 2002). In apple Ca deficiency problem is called "bitter pit" and characterized by occurrence of pitted small brown necrotic spots (Mengel and Kirkby, 2001). Also, Ca deficiency causes of the "tipburn" disorder in leaves of different vegetables (Saure, 1998).

2.2 Biochemical Functions of Calcium in Plants

A wide variety of diverse biochemical functions are initiated by or associated with changes in cellular Ca. Calcium is particularly important for structural stability and functional integrity of biological membranes and tissues. It is well known that an adequate supply of Ca maintains membrane integrity and contributes to ion selectivity (Marschner, 1995; Grattan and Grieve 1999; Mengel and Kirkby, 2001). Calcium binds as pectate in middle lamella of the cell wall for strengthening of the cell walls and plant tissues. The proportion of Ca pectate in the cell wall is also important for the susceptibility of the tissues to fungal and bacterial infections (Marschner 1995; Lamb and Dixon, 1997). The concentration of the Ca in the cytosol is extremely low and maintained in the range of 0.1-1.2 μM of free Ca, but such low calcium concentrations are so essential for various reasons, such as for the functioning of certain key enzymes including ATPases at the plasma membrane of roots and plasma membrane-bound NADPH oxidases (Marschner 1995; Keller et al., 1998).

Calcium has been found to be involved in the regulation of various responses of plants to biotic stresses caused by pathogen attacks (Yang and Poovaiah, 2002). An indispensable role of Ca in plant cells during the plant response to microbial or elicitor attack is well established (Grant et al., 2000; Sagi and Fluhr, 2001). During pathogen attacks, a membrane-bound enzyme resembling the neutrophil NADPH oxidase was identified to contribute to the pathogen-induced oxidative burst in plants by generating $O_2^{\cdot-}$ which leads indirectly to the generation of ROS (reactive oxygen species) in plants. Presently it is widely accepted that NADPH oxidases are the major source of ROS produced during the oxidative burst in plants (Mendy et al., 1996; Lamb and Dixon,

1997; Sagi and Fluhr, 2001). Therefore, communication between Ca and ROS production in pathogen attack is mediated mostly through by NADPH oxidases. The activity of NADPH oxidase, thus the production of both $O_2^{\cdot-}$ and H_2O_2 is enhanced in the presence of Ca through either by the Ca binding domains (EF hands) located on the NADPH subunit of $gp91^{phox}$ or indirectly by activating NAD kinase activity (Keller et al., 1998; Torres et al., 1998; Sagi and Fluhr, 2001).

Although Ca is involved in plant responses to biotic stresses, it also regulates the plant responses to environmental stresses, including heat, and water. Exogenously applied Ca alleviates heat injury and water stress by regulation of antioxidant activities such as GR, AP, and CAT, decreasing the membrane lipid peroxidation and helps plant cells to better survive during stress conditions (Jiang and Huang, 2001; Nayyar and Kaushal, 2002a; Fu and Huang, 2003).

2.3 Generation and Detoxification of ROS in Plants

As indicated above, environmental stress factors such as nutrient deficiencies, extreme temperatures and drought disrupt cellular homeostasis leading to enhanced production of ROS. Under such conditions the antioxidant enzyme activities can be suppressed causing peroxidation of cellular membranes (Foyer et al., 1997; Asada, 1999). ROS can be highly toxic even at low concentrations and can damage many important cellular components, such as chlorophyll, membrane lipids, protein SH-groups, DNA, and RNA (Halliwell and Guttridge, 1985).

Plants have evolved various enzymatic and non-enzymatic mechanisms to minimize the damaging effects of ROS produced by environmental stresses. Even under normal growth conditions, the production of ROS is unavoidable. It is estimated that under non-stressed conditions, up to 20 % of photosynthetic electrons can be used in activation of molecular O_2 and, this rate is increased when plants suffer from an environmental stress factor such as drought, salinity and nutrient deficiencies (Foyer et. al., 1997; Cakmak, 2000). ROS are not only produced during photosynthesis. There

are further cell organelles or metabolic processes producing ROS, such as during mitochondrial electron transport, photorespiration, and electron transport process on membranes. ROS must be effectively detoxified especially at the sites where they can cause irreversible damages to cellular membranes, chlorophyll and DNA. Plant cells are well equipped against ROS. Reactive oxygen species are effectively scavenged by non-enzymatic detoxification mechanisms (e.g., vitamin C, α-tocopherol, carotenoids) and/or antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (AP), and glutathione reductase (GR) (Mittler, 2002).

In enzymatic defense mechanism, superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2 which regulates a low steady state concentration of $O_2^{\cdot -}$ and, thus, minimizes hydroxyl radical formation by the superoxide radical catalyzed Haber-Weiss reaction (Halliwell and Guttridge, 1985; Bowler et al., 1992; Scandalios JG, 1993; Alscher et al., 2002). As mentioned above, generation of $O_2^{\cdot -}$ in chloroplasts is stimulated when plants are exposed to environmental stresses that limit photosynthetic CO_2 fixation, thus intensifies electrons flow to O_2 with concomitant generation of $O_2^{\cdot -}$ and other reactive oxygen species (Foyer et al., 1997; Asada, 1999). Hydrogen peroxide is eliminated by catalase and peroxidases (Halliwell and Guttridge, 1985; Foyer et al., 1994; Bolwell and Wojtaszek, 1997). Catalases are peroxisomal enzymes that, in contrast to peroxidases, do not require a reducing substrate for their activity.

In plant cells, an alternative and more effective detoxification mechanism against H_2O_2 also exists in chloroplast and cytosol, called "ascorbate-glutathione pathway" (Figure 2.1). As described by Sharma and Davies (1997) and Asada (1999), in this detoxification mechanism H_2O_2 is reduced to H_2O by ascorbate peroxidase (AP) and ascorbate is regenerated by the "ascorbate-glutathione cycle", involving monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase. Ascorbate is first oxidized to monodehydroascorbate by AP. Monodehydroascorbate is converted to ascorbate. The enzyme responsible for conversion of monodehydroascorbate to ascorbate is monodehydroascorbate reductase. When conversion of monodehydroascorbate to ascorbate does not take place quickly, then monodehydroascorbate will spontaneously disproportionate into ascorbate and

dehydroascorbate. Dehydroascorbate recycles ascorbate with the contribution of reduced glutathione that is generated through the action of glutathione reductase in a NADPH-dependent reaction (Cakmak, 1994; Foyer et al., 1994). The ascorbate-glutathione pathway is also present in cytosol and has a crucial importance for detoxification of H_2O_2 produced in seeds during germination (Cakmak et al., 1993).

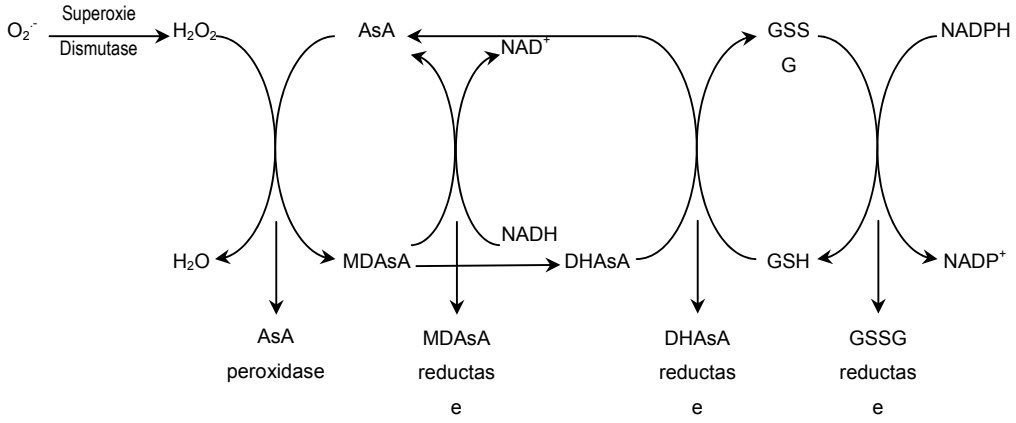

Figure 2.1 The ascorbate-glutathione pathway operating mainly in chloroplast to detoxify H_2O_2. MDAsA: monodehydroascorbate, DHAsA: dehydroascorbate, GSSG: oxidized glutathione, GSH: reduced glutathione

Ascorbic acid, glutathione, and α-tocopherol have each been shown to act as critical antioxidants playing a central role in the detoxification of ROS in plant cells under various stress conditions. They can either directly react with ROS and remove them, or contribute to enzyme-catalyzed detoxification reactions (Halliwell and Guttridge, 1985; Noctor and Foyer, 1998). Ascorbate is a major primary antioxidant, reacting directly with hydroxyl radical ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$). It indirectly eliminates H_2O_2 through the activity of ascorbate peroxidase (AP) during the ascorbate-glutathione cycle (Halliwell and Guttridge, 1985; Foyer at al., 1994; Foyer et al., 1997; Noctor and Foyer, 1998; Conklin, 2001). Ascorbate also acts as a reductant (electron donor) in the regeneration of α-tocopherol. Another important soluble antioxidant is glutathione, which is capable of detoxifying $^1O_2$ and $OH^{\cdot}$. Glutathione protects thiol groups in enzymes, and involves in α-tocopherol and ascorbate regeneration through the glutathione-ascorbate cycle (Foyer et al., 1994; Noctor and Foyer, 1998; Niyogi, 1999).

The levels of enzymatic and non-enzymatic antioxidant defense mechanism are affected by different factors, especially by mineral nutritional status of plants. For example, under Mg deficiency antioxidant mechanisms are generally activated and showed impressive increases (Cakmak and Marschner, 1992). By contrast, B deficiency decreased glutathione reductase and ascorbic acid levels (Cakmak and Römheld, 1997) and Zn deficiency depressed SOD activity (Cakmak, 2000).

Studies dealing with effect of Ca deficiency on antioxidant enzymes are very rare. In tomato plants, Ca deficiency was found to decrease SOD activity and levels of ascorbic acid and α-tocopherol (Schmitz-Eiberger et al., 2002). Because of the limited research on the effects of Ca deficiency on antioxidative defense mechanism and also NADPH oxidase, in this MSc work, we focused on measurement of activity of $O_2^{\cdot-}$ generating NADPH oxidase and antioxidative defense mechanisms in bean root cells grown by different Ca treatments.

3 MATERIALS AND METHODS

3.1 Plant Material and Growth Conditions

Bean plants (*Phaseolus vulgaris*, cultivar Nassua) were grown in growth camber under controlled environmental conditions (light/dark regime: 16/8 h at 20/18˚C, relative humidity: 65-75 %, photon flux density: 380 μmol m^{-2} s^{-1}). Seeds were first sterilized with 1% (w/v) calcium hypochlorite for 10 min, and then sown in perlite moistened with saturated $CaSO_4$ solution and finally kept in the dark for 5 days. The seedlings were then transferred to 2.5 L plastic pots containing aerated nutrient solution. The composition of the nutrient solution was as follows: 1mM $MgSO_4.7H_20$, 0.9 mM K_2SO_4, 0.2 mM KH_2PO_4, 10^{-6} M H_3BO_3, $2x10^{-7}$ M $MnSO_4.H_2O$, 10^{-6} M $ZnSO_4.7H_20$, $2x10^{-7}$ M $CuSO_4.5H_2O$, $2x10^{-8}$ M $(NH_4)_6Mo_7O_{24}.4H_20$, and 10^{-4} M FeEDTA ($C_{10}H_{12}FeN_2NaO_8$). After transfer of seedlings into the nutrient solution, all plants were supplied adequately by Ca. Calcium was supplied at concentration of 2 mM in the form of $Ca(NO_3)_2.4H_2O$ for the first 3 days After 3 days, parts of plants were transferred into a nutrient solution containing different levels of deficient Ca supplies (50, 100 and 250 μM Ca supply). The concentrations and duration of low Ca supplies were indicated in the legend of relevant figures and tables. For control plants (Ca-sufficient plants) Ca was always supplied as 2 mM $Ca(NO_3)_2.4H_2O$.

Plants were harvested after 5 days growth in low Ca supplied nutrient solution. At harvest, roots and shoots were separated. Roots were rinsed with 0.1 mM EDTA for about 10 min to remove surface adsorbed Ca and then deionised water throughly. Then, roots and shoots were dried at 70 ˚C for determination of dry matter production and Ca

concentration. For the analysis of enzymes and antioxidants, only roots were sampled and stored in liquid nitrogen until analysis.

3.2 Methods

3.2.1 Dry Matter Production and Measurements of Calcium Concentration

Shoot and root dry matter production was determined after drying plant samples at 70 ˚C. Approximately 0.2 g ground samples were ashed at 500 ˚C for 8 h and then dissolved in 3.3 % HCl. The concentration of Ca was determined by atomic absorption spectrometry (AAS). Calcium measurements in plant materials were checked against certified Ca values in different reference plant materials obtained from the National Institute of Standards and Technology (Gaithersburg, USA).

3.2.2 Determination of Non-Protein SH-Groups

The levels of non-protein sulfhydryl groups were determined colorimetrically using 5-5'-dithiobis- (2 nitro benzoic acid) (DTNB) following extraction of approximately 0.5 g fresh root sample with 5 % meta-phosphoric acid as described in Cakmak and Marschner (1992). The reaction mixture contained 0.5 ml aliquot of the 5000 g supernatant, 2.5 ml 150 mM phosphate buffer (pH 7.5) containing 5 mM EDTA and 0.5 ml 6 mM DTNB. After incubation at room temperature for 20 min, the color produced was measured at 412 nm using reduced glutathione as a standard in the range of 0 to 100 µg ml^{-1}.

3.2.3 Determination of Total Ascorbate Levels

Total ascorbate was determined according to Cakmak and Marschner (1992) with some modifications. Usually 0.5 g root samples were extracted with 5 ml 5 % *meta*-phosphoric acid and centrifuged at 5000 g for 30 min. Total ascorbate (AsA + DAsA) was measured after the reduction of DAsA (oxidized ascorbic acid) to AsA (reduced ascorbic acid) with DTT (1,4 dithiothreitol). The reaction mixture for total ascorbate contained 0.2 ml aliquot of the 5000 G supernatant, 0.5 ml 150 Mm phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.1 ml 10 mM DTT (dithioothreitol) and 0.1 ml 0.5 % *N*-ethylmaleimide (NEM) to remove excess DTT. In reaction mixture the color was developed after addition of the following reagents: 0.4 ml 10 % TCA, 0.4 ml 44 % *ortho*-phosphoric acid, 0.4 ml 4 % 2,2'-bipyridine in 70 % ethanole, and 0.2 ml 3 % $FeCl_3$. Then, the mixture was incubated at 40 ˚C for 40 min and the color produced was read at 525 nm using L(+) ascorbic acid as a standard in the range of 0 to 100 µg ml^{-1}.

3.2.4 Assays of Antioxidative Enzymes

For the preparation of the root extracts, root tissue was homogenized using mortar and pestle with 6-8 ml of ice-cold 50 mM phosphate extraction buffer (pH 7.6) containing 0.1 mM Na-EDTA, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.3 % (w/v) polyvinylpyrrolidone. The homogenized samples were first centrifuged at 5000 g for 15 min (pellet discarded) and at 60,000 g for 120 min. The resultant supernatant was used for assay of enzymes. With the exception of SOD, all enzyme activities were measured in a final volume of 1 cm^3 at 25 ˚C.

3.2.4.1 Ascorbate Peroxidase Activity

Ascorbate peroxidase (AP) activity was determined according to Cakmak (1994) by following the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM cm^{-1})

in a 1 ml of reaction mixture containing 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 12 mM H_2O_2, 0.25 mM ascorbic acid and the enzyme extract. Correction was done for the very low, non-enzymatic oxidation of ascorbic acid by H_2O_2.

3.2.4.2 Glutathione Reductase Activity

Activity of glutathione reductase (GR) was measured according to Cakmak and Marschner (1992) by monitoring the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM cm^{-1}). The reaction mixture (1 ml) contained 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 0.5 mM oxidized glutathione (GSSG), 0.12 mM NADPH, and the enzyme extract. Corrections were made for the background absorbance at 340 nm without NADPH.

3.2.4.3 Superoxide Dismutase Activity

Activity of superoxide dismutase (SOD) was assayed by a photochemical method described in Cakmak and Marschner (1992) and based on a SOD-inhibitable reduction of nitro blue tetrazolium chloride (NBT) by superoxide radicals. Assays were carried out under illumination in growth chamber. For the SOD assay, the reaction medium (5 ml) was consisted of 50 mM phosphate buffer (pH 7.6), 0.1 mM Na-EDTA, enzyme extracts (50-150 µl), 50 mM Na_2CO_3 (pH 10.2), 12 mM L-methionine, 75 µM p-nitro blue tetrazolium chloride (NBT) and 2 µM riboflavin was maintained in a glass vials. The assay was illuminated under a light intensity of about 400 µE m^{-2} s^{-1} for 10 min. The amount of enzyme extract that caused a 50 % decrease in the SOD-inhibitable NBT reduction was defined as 1 unit.

3.2.4.4 Catalase Activity

Catalase (CAT) activity was determined by following the consumption of H_2O_2 at 240 nm (extinction coefficient 39.4 mM cm^{-1}). The reaction medium (1 ml) contained 50 mM phosphate buffer (7.6), 0.1 mM EDTA, 0.1 ml 100 mM H_2O_2 and the enzyme extract (Cakmak and Marschner, 1992).

3.2.5 Measurement of NADPH-dependent $O_2^{\cdot-}$ Generation

The assay of NADPH-dependent $O_2^{\cdot-}$ generation in the 60.000 g supernatants of root extracts was carried out as described by Cakmak and Marschner (1988) with some modifications. In the present study we used NBT instead of cytochrome c, and we measured the rate of SOD-inhibitable NBT reduction in the presence of NADPH. Reaction mixtures in reference and sample cuvettes contained 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 1µM KCN, 0.5 mM NBT, root extract and 50 µM NADPH in a total volume of 1 ml. Superoxide dismutase was added to the reference cuvette to a final concentration of 25 µg ml^{-1} (100 U/100 µl). After a 1 min preincubation the reaction was started by the addition of NADPH to both cuvettes, and the absorbance changes at 550 nm was followed usually for 5 min. Rates of $O_2^{\cdot-}$ -generation was calculated using an extinction coefficient of 12.8 mM^{-1} cm^{-1}. Under same conditions, NADPH oxidation was measured at 340 nm, except that NBT was omitted from the reaction mixture. Rate of NADPH oxidation was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

4 RESULTS

4.1 Calcium Deficiency Symptoms and Root and Shoot Dry Matter Production

The first visible characteristic reaction of bean plants to Ca deficiency was the decrease in root elongation (Figure 4.1). Reduction in root elongation was observable within one day following transfer of Ca-adequate plants (2000 μM Ca supply) into Ca-deficient (50 μM Ca supply) nutrient solution. The reduction in root elongation was followed by the development of brownish color along the roots (Figure 4.2). The roots of bean plants supplied with adequate Ca did not turn to brown color and not fail to elongate. Interestingly, during decreases in root elongation and brownish color formation on the roots, there was no change in shoot growth and no leaf symptoms of Ca deficiency at least within the first 2-3 days of Ca deficiency treatments.

Accordingly, root growth was much more affected by Ca deficiency than shoot growth (Figure 4.1 and 4.2). As presented in Table 4.1, deficient supply of Ca distinctly reduced root growth but caused very little effect on shoot growth. For example, by decreasing Ca supply in nutrient solution from 2000 to 50 μM, the root dry weight decreased by 36 % while the shoot dry weight decreased only by 5 % (Table 4.1). Such differential response of root and shoot growth to low Ca supply caused a higher shoot/root dry weight ratio in Ca deficient plants (Table 4.1).

Re-supply of a sufficient Ca (2000 μM) to Ca-deficient plants enhanced dry matter production of plants within 2 days (Table 4.2). Increases in dry matter production due to Ca re-supply to Ca-deficient plants became more distinct with time especially for

the root dry matter production. The increase in root dry weight by Ca re-supply was around 36 % for the 48 h re-supply treatment while for shoots this increase was only 10 % (Table 4.2). Both root and shoot dry weight of plants was not affected within 8 hours re-supply of Ca.

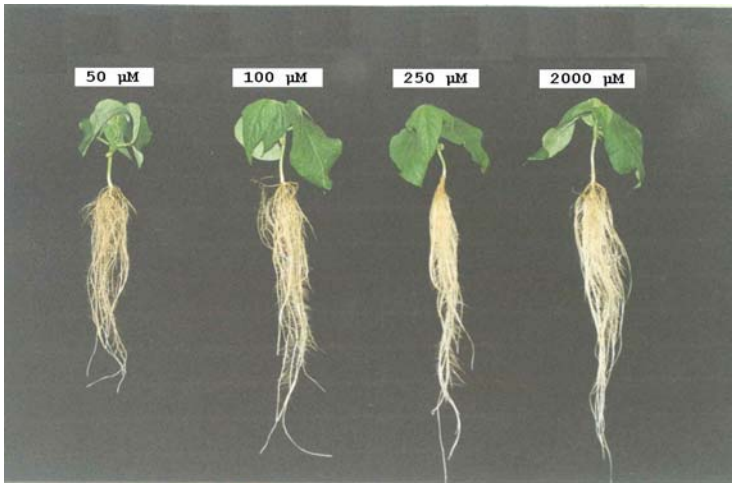

Figure 4.1 Effect of increasing Ca supply on shoot and root growth of bean plants grown for 13 days in nutrient solution. Low Ca doses (50-250 µM) were applied when plants were 8 days old in nutrient solution.

Figure 4.2 Inhibition of root elongation and formation of brownish color along the roots of Ca-deficient (50 µM) bean plants.

Table 4.1 Effect of increasing Ca supply on shoot dry weight and shoot to root ratio in bean plants grown for 13 days in nutrient solution. Plants were exposed to low Ca (50-250μM) treatments when they were 8 days old. The data represent mean±SD from four independent replications.

Ca supply	Shoot Dry weight	Root Dry weight	Shoot/Root
(μM)	(g plant^{-1})		
2000*	0.73±0.07	0.23±0.02	3.2
250	0.71±0.07	0.20±0.01	3.6
100	0.69±0.04	0.19±0.01	3.6
50	0.69±0.05	0.15±0.02	4.7

*Adequate Ca supply.

Table 4.2 Shoot and root dry weights and shoot to root ratio in 13-days-old bean plants treated with sufficient (2000 μM) and/or deficient (50 μM) Ca for different times. Resupply of Ca to Ca-deficient plants was realized at concentration of 2000 μM for 8h, 24h and 48h before the harvest of plants. The data represent mean±SD from four independent replications

Ca supply	Shoot Dry weight	Root Dry weight	Shoot/root
(μM)	(g plant^{-1})		
2000	0.81±0.01	0.20±0.03	4.2
50	0.71±0.05	0.14±0.02	5.0
50+ 8h Ca Resupply*	0.72±0.06	0.14±0.01	5.0
50+24h Ca Resupply	0.76±0.01	0.16±0.03	4.8
50+48h Ca Resupply	0.79±0.07	0.19±0.02	4.2

* Ca-resupply to deficient plants: 2000 μM.

4.2 Calcium Concentration and Content

There was a close relationship between the amount of Ca in nutrient solution and Ca concentration of bean plants. With increasing supply of Ca from 50 μM to 200 μM Ca concentration of shoot was increased by a factor of 5 (Table 4.3). When compared to shoot, root had much less Ca concentration indicating very high mobility and transport of Ca from root into shoot. Similarly, total amount of Ca (Ca content) per plant was markedly enhanced by increasing Ca supply (Table 4.3).

Re-supply of Ca to Ca-deficient plants enhanced Ca concentration of plants very rapidly, especially in shoots (Table 4.4). Already within 8 hours of Ca re-supply, shoot and root concentrations of Ca were clearly increased. This increase was around 2-fold in root following 8 h re-supply of Ca. However, there was no further increase in root Ca concentration after 24 h or 48 h Ca re-supply. In contrast to roots, Ca concentrations of shoots progressively increased with duration of Ca re-supply to Ca-deficient plants (Table 4.4).

It seems that most of the re-supplied Ca was taken up and rapidly transported into shoots. This can also be seen in shoot content of Ca. During 48 h re-supply of Ca, shoot content was increased by around 8-fold, while for roots this increase was only 2.5 fold (Table 4.4).

Table 4.3 Effect of increasing Ca supply on concentration and content (total amount) of Ca in shoots and roots of bean plants grown for 13 days in nutrient solution. Plants were exposed to low Ca supplies (50-250μM) when they were 8 days old in nutrient solution. The data represent mean±SD from four independent replications.

Ca supply	Ca concentration	Ca content
(μM)	(%)	(mg plant^{-1})
	Shoot	
2000	1.54±0.10	11.14±0.74
250	0.59±0.04	4.23±0.52
100	0.41±0.03	2.83±0.18
50	0.29±0.02	2.03±0.26
	Root	
2000	0.27±0.01	0.61±0.03
250	0.16±0	0.31±0.10
100	0.13±0.01	0.25±0.02
50	0.12±0.01	0.18±0.01

Table 4.4 The concentration and content of Ca in shoots and roots of 13-days-old bean plants treated with deficient doses of Ca. Ca-deficient plants (50 μM) were exposed to Ca for 8h, 24h and 48h at concentration of 2000 μM before the harvest. The data represent mean±SD from four independent replications.

Ca supply	Ca concentration	Ca content
(μM)	(%)	(mg plant^{-1})
	Shoot	
2000	2.03±0.10	16.15±0.92
50	0.23±0.02	1.64±0.10
50+ 8h Ca Resupply*	0.32±0.04	2.28±0.29
50+24h Ca Resupply	0.43±0.14	3.35±1.41
50+48h Ca Resupply	1.48±0.01	11.63±1.12
	Root	
2000	0.23±0.01	0.46±0.08
50	0.08±0	0.12±0.02
50+ 8h Ca Resupply*	0.16±0	0.23±0.02
50+24h Ca Resupply	0.14±0.03	0.21±0.05
50+48h Ca Resupply	0.16±0.01	0.30±0.03

*Ca-resupply to deficient plants: 2000 μM

4.3 Non-Protein SH-Groups

The concentration of non-protein SH-groups showed a high sensitivity of decreasing Ca supply (Figure 4.3A). With decreasing Ca supply a rapid decline in concentration of SH-groups was found in roots. The concentration of SH groups reduced from 66 to 27 mg g^{-1} FW by decreasing Ca supply from 2000 to 50μM (Figure 4.3A). This decrease was especially found at 100 and 50 μM Ca applications. As the root growth was severely decreased by Ca supply, the total amount of SH-groups per root could be much lower when compared to Ca-sufficient plants.

4.4 Ascorbic Acid Concentration

In contrast to the non-protein SH-groups, varied Ca supply did not result in a consistent effect on ascorbic acid concentration of roots (Figures 4.3B). Decreasing Ca supply tended to increase ascorbic acid concentration, but this effect was not clear due to large standard deviation at 100 μM Ca supply (Figure 4.3B). The reason for irregular changes of ascorbic acid levels in Ca-deficient roots could not be understood.

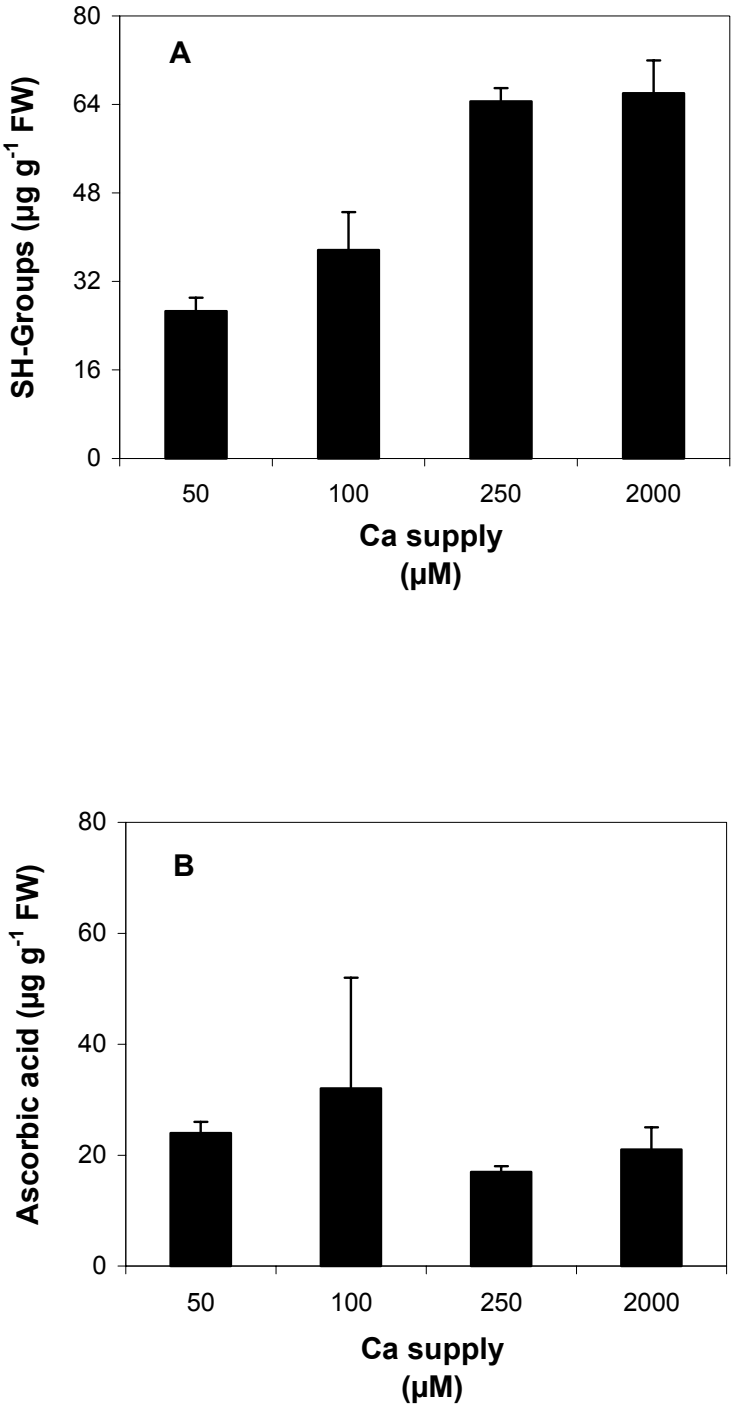

Figure 4.3 Changes in the concentration of non-protein SH-groups (above) and ascorbic acid concentrations (below) in roots of 13 d-old bean plants exposed to increasing Ca supply from 50 µM to 2000 µM. Bars represent the mean±SD of four independent replications.

4.5 Glutathione Reductase

Bean roots responded to enhanced Ca supply with a marked increase in the activity of glutathione reductase (GR) (Figure 4.4). A reduction in the activity of GR was already detected starting from the 250 µM Ca supply and became more distinct at the 50 µM Ca treatment. The decrease in GR activity by severe Ca deficiency was approximately 3-fold (Figure 4.4).

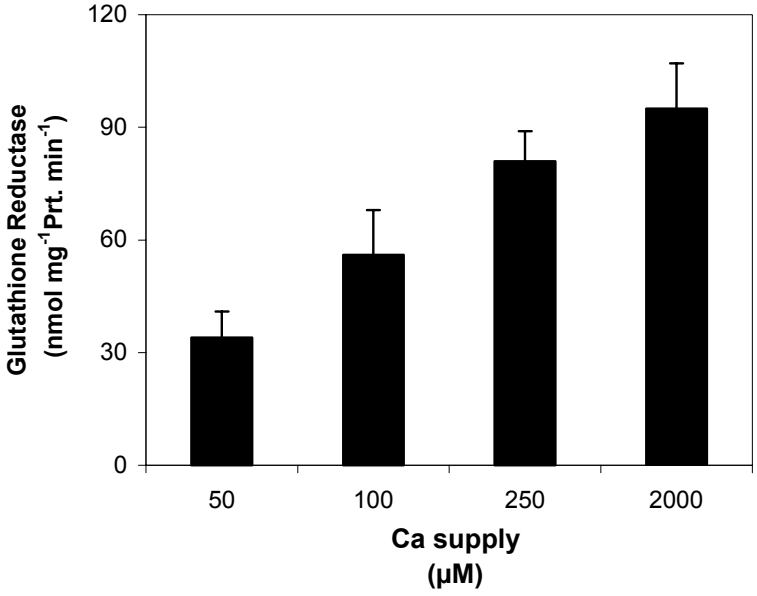

Figure 4.4 Changes in the activity of glutathione reductase (GR) in roots of 13 d-old bean plants exposed to increasing Ca supply from 50 µM to 2000 µM. Bars represent the mean±SD of four independent replications.

4.6 Ascorbate Peroxidase

Similar to ascorbic acid concentration also ascorbate peroxidase (AP) activity was not affected by different Ca treatments (Figure 4.5). There was a slight tendency to decrease in AP activity by increasing Ca supply.

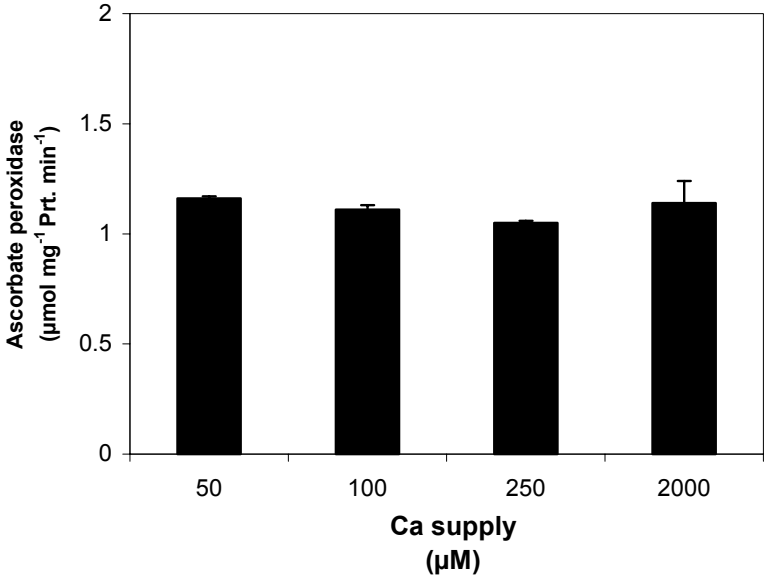

Figure 4.5 Changes in the activity of ascorbate peroxidase (AP) in roots of 13 d-old bean plants exposed to increasing Ca supply from 50 µM to 2000 µM. Bars represent the mean±SD from four independent replications.

4.7 Catalase

Also catalase activity did not show any consistent change by increasing Ca supply (Figure 4.6).

4.8 Superoxide Dismutase

Varied supply of Ca did not affect the activity of superoxide dismutase (SOD) in roots of bean plants (Figure 4.6).

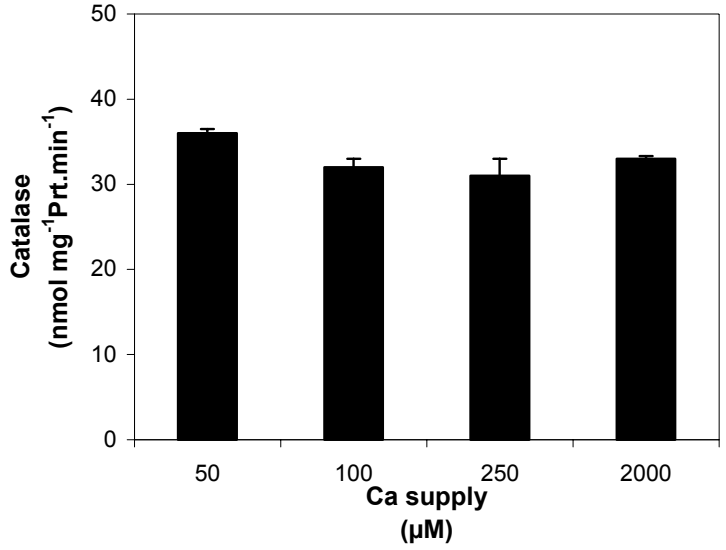

Figure 4.6 Changes in the activity of catalase (CAT) in roots of 13 d-old bean plants exposed to increasing Ca supply from 50µM to 2000 µM. Bars represent the mean±SD from four independent replications.

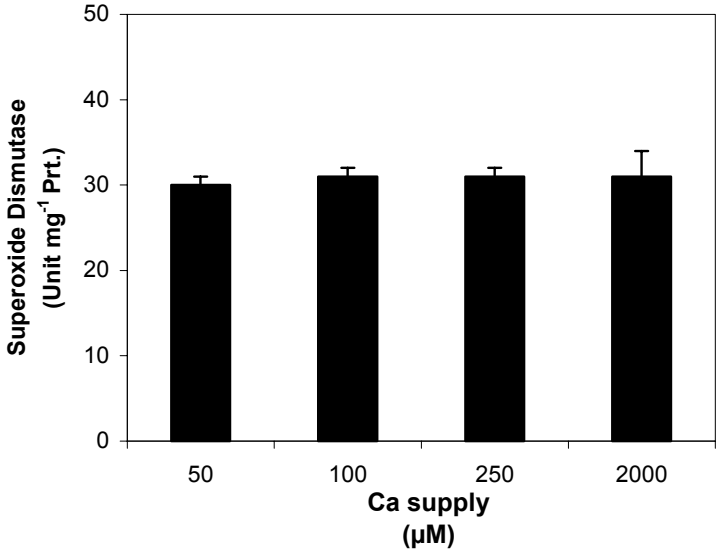

Figure 4.7 Changes in the activity of superoxide dismutase (SOD) in roots of 13 d-old bean plants exposed to increasing C supply from 50 µM to2000 µM. Bars represent the mean±SD from four independent replications.

4.9 NADPH-dependent $O_2^{\cdot -}$ Generation

Calcium deficiency depressed the activity of NADPH oxidase and NADPH dependent $O_2^{\cdot -}$ generation in the cytosol fraction of bean roots (Table 4.5). The decreases were more distinct in the case of NADPH oxidase. With the lowest Ca supply (50 μM) NADPH oxidase activity of roots was decreased by around 50 % while NADPH-dependent $O_2^{\cdot -}$ generation showed a decrease of around 30 %. There was a very close positive relationship between Ca concentration in nutrient solution and activity of NADPH oxidase (Table 4.5). Activities of both NADPH oxidase and NADPH-dependent $O_2^{\cdot -}$ generation were sensitive to treatment with DPI (Diphenylene iodonium), an inhibitor of $O_2^{\cdot -}$ generating NADPH oxidase activity. DPI was effective to depress activity of enzymes by around 50 % (data not shown).

Table 4.5 Changes in the activity of NADPH-dependent $O_2^{\cdot -}$ generation and NADPH oxidase in cytosolic fraction of roots of 13 d-old bean plants exposed to increasing Ca supply from 50 μM (deficient) to 2000 μM (sufficient). The data represent the mean±SD from four independent replications.

Ca supply	NADPH-dependent $O_2^{\cdot -}$ generation ($\mu mol\ mg^{-1}\ prt.\ min^{-1}$)	NADPH oxidase activity ($\mu mol\ mg^{-1}\ prt.\ min^{-1}$)
2000	6.6 ± 1.2	33.6 ± 9.2
250	5.5 ± 0.6	25.3 ± 9.9
100	5.5 ± 1.2	20.0 ± 7.7
50	4.7 ± 1.0	17.6 ± 2.6

In the second experiment, effect of Ca deficiency on $O_2^{\cdot-}$ generating NADPH oxidase was studied over time and monitored during 5 days of Ca deficiency stress (Table 4.6). Also in this experiment with the severity of Ca deficiency stress, $O_2^{\cdot-}$ generation rate and NADPH oxidase activity showed a very clear decrease. These decreases were also closely associated with appearance of brownish color formation along the roots. These results together with those presented in Table 4.5 indicate the involvement of Ca in NADPH-dependent $O_2^{\cdot-}$ generation process.

Table 4.6 Changes in activities of NADPH-dependent $O_2^{\cdot-}$ generation and NADPH oxidase in cytosolic fraction of root extracts over 5 days of Ca deficiency stress (50 μM). Low Ca supply was started following the growth of plants at 2000 μM Ca for 3 days. The data represent the mean±SD from four independent replications.

Days of deficiency*	NADPH-dependent $O_2^{\cdot-}$ generation ($\mu mol\ mg^{-1}\ prt.\ min^{-1}$)	NADPH oxidase activity ($\mu mol\ mg^{-1}\ prt.\ min^{-1}$)
0**	8.8 ± 0.6	30.2 ± 8.1
2	6.9 ± 0.4	25.1 ± 7.4
3	6.4 ± 0.6	23.3 ± 1.2
4	5.7 ± 1.1	19.0 ± 4.2
5	4.6 ± 0.4	20.9 ± 3.8

** Control plant with adequate Ca supply (2000 μM)

* Control plant (2000 μM) transferred into solution containing 50 μM Ca

Resupply of 2000 μM Ca to Ca-deficient plants was highly effective to reverse the decrease in both NADPH oxidase and NADPH-dependent $O_2^{\cdot-}$ generation in roots (Table 4.7). As found in other experiments (Tables 4.5 and 4.6), at low Ca supply (50 μM) activities of NADPH oxidase and $O_2^{\cdot-}$ generation were severely depressed when compared to sufficient Ca supply (Table 4.7). Resupply of Ca (2000 μM) to Ca-

deficient plants enhanced activities of $O_2^{\cdot -}$ generation and NADPH oxidase already within 8 hours. This increase in activities by resupplied Ca continued and became pronounced after 48 h of Ca resupply, especially in the case of NADPH oxidation. Within 8 h of Ca resupply, NADPH oxidase activity was enhanced by 2.3 fold (Table 4.7).

Table 4.7 Effect of varied Ca supply on NADPH-dependent $O_2^{\cdot -}$ generation and NADPH oxidase in cytosolic fraction of root cells. Resupply of Ca to Ca-deficient plants was started after 5 days growth at 50 μM Ca supply. The data represent the mean±SD from four independent replications.

Ca supply	NADPH-dependent $O_2^{\cdot -}$ generation ($\mu mol\ mg^{-1}\ prt.\ min^{-1}$)	NADPH oxidase activity ($\mu mol\ mg^{-1}\ prt.\ min^{-1}$)
2000	11.2 ± 0.8	27.9 ± 6.6
50	5.9 ± 0.4	6.5 ± 2.5
50+ 8h Ca Resupply*	6.3 ± 0.7	15.1 ± 5.1
50+24h Ca Resupply	6.6 ± 0.4	13.6 ± 2.7
50+48h Ca Resupply	8.0 ± 0.4	20.5 ± 2.6

* Adequate Ca (2000 μM) resupplied to Ca deficient plants (50 μM)

5 DISCUSSION

5.1 Leaf Symptoms and Growth

When compared to shoot growth, root growth showed much higher sensitivity to Ca deficiency (Figure 4.1 and 4.2). The reason for higher sensitivity of roots to Ca deficiency can be related to the fact that Ca is essential for root elongation, density and length of the roots which are necessary for nutrient absorption (Juanin and Hofer, 1988; Marschner, 1995). Additionally, once Ca is deposited in older leaves it cannot be mobilized to the growing tips (root tips, shoot apex) and the rate of downward translocation of Ca is extremely low due to the fact that Ca is transported in only very small concentrations in the phloem (Marschner, 1995). When taken up by roots, Ca is very rapidly transported to shoot by xylem transport. Consequently roots contain much lower Ca than shoots (Tables 4.3 and 4.4). Therefore, in the case of resupply of Ca to Ca-deficient plants root growth was much stronger and earlier affected than shoot growth (Table 4.2).

Shoot growth remained more or less the same during Ca deficiency stress under given conditions (Table 4.1). Therefore, shoot to root ratios showed an increase with increasing severity of Ca deficiency stress (Tables 4.1 and 4.2). A similar behavior of shoot and root growth was also found in Mg- and K-deficient plants and attributed to lower transport of carbohydrates from shoot into root (Cakmak et al., 1994; Cakmak, 1994). There was no clear relationship between the shoot dry matter production and

shoot Ca concentration in bean plants. In the present study, shoots contained 81 % less Ca compared with the control plants but their dry matter production remained almost the same when compared with control plants (Table 4.1 and 4.3). It seems that shoots may accumulate (store) more Ca than they need for their growth, and thus, when they are exposed to Ca deficiency conditions, they may provide physiologically available Ca from their stocks. Consequently, their dry matter production may not be so affected in comparison to root growth.

With the begin of Ca deficiency treatment; there was a brown coloring formation along the roots. This may be probably due to the brown melanin compounds resulting from polyphenol oxidation, which are associated with the deficient tissues. DeKock et al. (1975) claimed that in tissues containing adequate amounts of Ca, this oxidation is inhibited by the chelation of the phenolic compounds by Ca. A similar dark-browning of roots is also caused by B deficiency, and accumulation and oxidation of phenols is considered as a major reason for dark-browning of B-deficient roots (Cakmak and Römheld, 1997). Also, Seling et al (2000) claimed that in potato plants containing inadequate amount of Ca tended to enhance the activity of polygalacturonase, which could control the breakdown of pectic polysaccharides in the cell wall and, thus, might lead to the occurrence of browning color formation along the roots.

5.2 Calcium Uptake

Besides the differences in severity of Ca deficiency symptoms between shoots and roots, bean plants had different Ca concentrations in shoots and roots (Table 4.3). As indicated above, shoots accumulated more Ca than roots under given conditions, especially at adequate Ca supply (Table 4.3). Following exposure of plants to low Ca supply, shoots tended to decrease Ca uptake more clearly than the roots when compared with control plants (Table 4.3). On the other hand, root growth was more distinctly inhibited than shoot growth, and the concentration of Ca in roots was lesser than the shoot Ca concentration. As mentioned before, it seems likely that differential sensitivity

of shoots and roots to Ca deficiency may be related to the concentrations or contents of Ca accumulated in shoots and roots.

When re-supplied to deficient plants Ca was taken up and accumulated at greater amounts in shoots than roots (Table 4.4). During the adequate re-supply of Ca to Ca-deficient plants, shoots tended to take up 3-fold more Ca than roots. Despite huge amount of Ca uptake and accumulation in shoot after resupply of Ca to Ca-deficient plants shoot dry matter production of Ca-deficient plants was increased by only 11 % (Table 4.4). This indicates again that rates of shoot growth and Ca accumulation in bean plants are not proportional. It seems likely that irrespective of growth, bean can accumulate higher amount of Ca in shoot. After resupply of Ca to Ca-deficient plants, roots were not able to accumulate Ca in tissue in contrast to shoots (Table 4.4). This indicates that Ca taken up by roots was translocated exclusively to the upper plant organs via xylem, and Ca in shoot cannot be translocated into root tips from shoot apex by floem transport (Marschner, 1995).

5.3 Antioxidative Defense System

5.3.1 Non-Protein SH-Groups (Glutathione)

Exposure of plants to decreasing concentration of Ca resulted in a rapid decrease in concentration of non-protein SH-compounds (thiols) in roots of bean plants (Figure 4.3). The decrease in level of non-protein SH-compounds in Ca-deficient roots was around 61 % when compared to the control plants. Glutathione comprises the major fraction of the total non-protein SH-compounds in various plant species, for example 95 % in spruce needles, and more than 80 % in other different plant species (Grill et al., 1979; Maas et al., 1987). Therefore, the SH-groups measured in the roots can be ascribed to glutathione. Glutathione is a key factor of the ascorbate-glutathione detoxification pathway, as being the substrate of GR (Foyer et al., 1994). The decrease in SH-containing compounds (i.e., glutathione) may be responsible for reduced activity

of glutathione reductase (GR). As discussed below, GR activity is also depressed by Ca deficiency possibly due to reduced substrate availability (see section 5.3.3 and Fig. 4.3). Alternatively, reduced levels of SH-compounds can be related to their extensive use in oxidation process caused by Ca deficiency. It is well-known that SH-compounds are able to interfere with the oxidation of phenols, which leads to the formation of brown colors in tissues (Golan-Goldhirsh and Whitaker, 1984). Similarly, in potato plants Ca deficiency was found to be responsible for browning of roots, and the reason for tissue browning in Ca deficient plants was attributed to enhanced activity of cell wall-bound polygalacturonase (Seling et al., 2000). Enhancement in polygalacturonase activity is responsible for breakdown of pectic polysaccharides in cell wall and the degradation products of pectic substrates are involved in tissue browning. It seems highly possible that Ca deficiency is associated with oxidation of phenolic compounds, which in turn results in browning of tissue. As found in B-deficient plants extensive oxidation of phenolics can be responsible for use of reducing compounds such as SH-containing compounds (Cakmak and Römheld, 1997). In future studies, a special attention should be paid to understanding the relationship between SH-compounds and phenol oxidation in Ca-deficient plants.

5.3.2 Ascorbic Acid

Despite the significant decline in concentrations of SH-containing compounds, roots showed irregular changes in ascorbic acid concentrations in response to varied Ca supply. The reason for such irregular changes in levels of ascorbic acid is not well understood. To our knowledge, there is no information in literature concerning the relationship between ascorbic acid and Ca deficiency in roots of plants. Ascorbic acid plays a major role for detoxification of H_2O_2 in ascorbate-glutathione pathway acting as a substrate for AP or involves in regeneration of reduced α-tocopherol (Conklin, 2001). The irregular changes of ascorbic acid concentration during Ca deficiency may due to the non-consistent formation of oxidative stress conditions. However, in tomato leaves Ca deficiency was found to be responsible for decreases in ascorbic acid concentration. This decrease was associated with decreases in α-tocopherol concentration and increase in lipid peroxidation (Schmitz-Eiberger et al., 2002).

5.3.3 Antioxidative Defense Enzymes

As with levels of SH-compounds, there was a particular decrease in levels of glutathione reductase (GR) activity in response to Ca deficiency. As compared with Ca-sufficient plant, the decrease in activity of glutathione reductase caused by Ca deficiency was about 64 % (Figure 4.4). By contrast, activities of other protective enzymes (AP, SOD and CAT) were not affected by Ca deficiency (figures 4.5, 4.6 and 4.7). Glutathione reductase (GR) is essential for maintenance of high concentrations of reduced glutathione, and directly involved in H_2O_2 detoxification (Figure 2.1; Foyer et al., 1994). Also glutathione reductase plays an important role in maintaining the cellular antioxidant/prooxidant ratio. The reason for decrease in GR by Ca deficiency is not well understood at present. The decrease in glutathione reductase (GR) by Ca deficiency might be critical because, glutathione reductase (GR) is one of the AsA-dependent H_2O_2 scavenging enzymes, thus, Ca-deficient plants might be so susceptible to peroxidative damage triggered by enhanced production of toxic O_2 species. Accordingly, Schmitz-Eiberger et al (2002) showed that Ca deficiency is associated with enhanced lipid peroxidation in tomato leaves. Heat stress-induced lipid peroxidation in arabidopsis was found to be highly sensitive to low Ca supply (Larkindale and Knight, 2002). In the case of sufficient Ca supply to plants grown at 40˚C lipid peroxidation was markedly inhibited and survival of plants at heat stress was significantly enhanced. The results indicate that Ca deficiency can induce oxidative damage in root cells, at least by affecting glutathione reductase and, thus, glutathione in roots. The reason for decreased GR activity could not be understood, and needs to be studied in future studies.

5.4 NADPH-dependent $O_2^{\cdot-}$ Generation

By generating superoxide radicals ($O_2^{\cdot-}$) the membrane-bound NADPH oxidases in plants are involved in different cellular processes in plants such as defense against pathogen attack or cell damage under abiotic stress. It is well documented that $O_2^{\cdot-}$ -generating NADPH-oxidases are activated by different biotic and abiotic stress

conditions. Cell damage caused by low temperature stress in plants has been found to be well related to increases in activity of $O_2^{\cdot-}$-generating NADPH-oxidase (Shen et al., 2000). Similarly, impairments in structural and functional integrity of root cell membranes under Zn deficiency (Cakmak and Marschner, 1988; Pinton et al., 1993) and Cu deficiency or toxicity (Quartacci et al., 2001) were related to enhanced activity of $O_2^{\cdot-}$-generating NADPH oxidase.

Impairments in structural integrity of cell membranes under Ca deficiency is a well-known phenomena (Marschner, 1995; Mengel and Kirkby, 2001). The results presented in Tables 4.5, 4.6 and 4.7 show that $O_2^{\cdot-}$-generating NADPH oxidase is not involved in Ca deficiency-induced impairments in membrane stabilization. In the present work, Ca deficiency caused a remarkable decrease in the activity of NADPH-dependent $O_2^{\cdot-}$ generation. The decrease in the $O_2^{\cdot-}$ generating activity with Ca deficiency was closely paralleled by decreased rates of NADPH oxidation (Table 4.5) suggesting that Ca might be involved in the generation of $O_2^{\cdot-}$ via an NADPH-dependent oxidase. Pretreatment of the root extracts with DPI (Diphenylene iodonium chloride) which is used as an inhibitor of NADPH oxidase in mammalian system (Babior, 1999), inhibited the activity of NADPH-dependent $O_2^{\cdot-}$ generation more or less 50 % indicating the production of $O_2^{\cdot-}$ via NADPH oxidases and not by other peroxidases. The decrease in the activity of NADPH oxidase and, thus NADPH-dependent $O_2^{\cdot-}$ generation during Ca deficiency can be due to the inactivation of Ca binding domains located on NADPH oxidase subunit ($gp91^{phox}$) (Keller et al., 1998; Torres 1998). Therefore, exposure of inadequate Ca supply might not be sufficient to activate the "EF-hand motifs" (Ca binding domains) located on the subunit of NADPH oxidase, thus causes a decrease in the generation of $O_2^{\cdot-}$ through NADPH oxidase. Re-supply of Ca to Ca deficient roots enhanced the activity of NADPH oxidase and NADPH-dependent $O_2^{\cdot-}$ generation, suggesting that Ca can stimulate the activity of NADPH oxidase through activating its subunit (Table 4.7). The decrease in the activity of NADPH oxidase in Ca-deficient roots may be also caused by the loss of membrane structure and integrity of root cells. NADPH oxidases are generally membrane-bound enzymes and any change in membrane structure can affect activity of NADPH oxidases.

Superoxide generating NADPH oxidases are closely involved in pathogen-induced oxidative stress in plants similar to neutrophil NADPH oxidases (Levine et al., 1994; Doke et al., 1996; Babior, 1999). Plants exposed to various pathogens develop adaptive mechanisms to resist or avoid pathogens attacked. The most well-known adaptive response is the hypersensitive reaction where reactive oxygen species (ROS) are the major component of this resistance mechanism operating against pathogens (Lamb and Dixon, 1997; Babior, 1999). These indicate that an adequate supply of Ca to plants is essential for protection of plants from pathogenic attack. Recently, Ca was shown to be essential for activation of $O_2^{\cdot-}$ generating NADPH oxidases in tobacco plants infected by tobacco mosaic virus (Sagi and Fluhr, 2001). Recently Lecourieux et al. (2002) showed that in *Nicotiana plumbaginifolia* cell suspension elicitors secreted by pathogens were effective to activate $O_2^{\cdot-}$ generating NADPH oxidases, and this activation was associated by enhanced cytosolic Ca concentration. Increases in cytosolic Ca concentrations caused by different environmental stress factors can induce $O_2^{\cdot-}$ generating NADPH oxidase and contribute physiological response of plants (Yang and Poovaiah, 2002). Abscisic acid (ABA) is increased as a consequence of water stress and involved in closing of stomatal pores. Increasing evidence suggest that $O_2^{\cdot-}$ generating NADPH oxidases are needed for ABA signaling effects (Murata et al., 2001; Jiang and Zhang, 2002). In well agreement with these results it has been shown that cold-induced stomatal closure needs high amount of Ca (Wilkinson et al., 2001). All these results together indicate that by affecting NADPH oxidase Ca nutritional status of plants may play critical roles in adaptive response of plants to environmental stress factors especially in development of resistance to pathogen attack and regulation of stomatal closure.

6 CONCLUSIONS

In the present study, the effects of varied Ca supply on shoot and root growth, activity of $O_2^{\cdot-}$-generating NADPH oxidase and antioxidative defense systems were investigated in roots of bean plants grown in nutrient solution under controlled climatic conditions

Calcium deficiency decreased root growth more severely than the shoot growth causing higher shoot to root ratio (Figures 4.1, 4.2 and Table 4.1). Irrespective of Ca supply shoots contained much more Ca than roots, particularly at sufficient Ca supply. At sufficient Ca supply shoots accumulated Ca up to 18-fold more than roots, while at deficient supply of Ca, shoots had 11-fold more Ca than roots. One possible reason of such distinct difference in Ca concentration between root and shoot might be ascribed to rapid transport of Ca into shoots via xylem by transpiration. According to Marschner (1995) Ca taken up by roots is very rapidly transported and accumulated in shoots because there is no resorption of Ca by xylem tissues in roots and no retranslocation of Ca from shoot into root via phloem.

Of the antioxidative defense systems studied in the present work only glutathione reductase (GR) and non-protein SH-groups (predominantly glutathione) were significantly affected by Ca deficiency. There was a marked decrease in both GR activity and SH levels in roots by Ca deficiency. The reason for such distinct decreases by Ca deficiency could not be understood. It seems likely that in Ca deficient roots with intense dark-brown formation, an enhanced oxidation of phenolic compounds by polyphenol oxidase (PPO) occurs causing accumulation of quinones, which are supposed to reduce levels of SH-containing compounds and inactivate GR activity (Cakmak and Römheld, 1997). The relationship between Ca nutrition and glutathione

metabolism is an important area for future research. In this regard a special attention should be paid to phenol metabolism.

Calcium was also essential for activity of $O_2^{\cdot-}$-generating NADPH oxidase. Deficient supply of Ca resulted in distinct decreases in both NADPH oxidation and $O_2^{\cdot-}$-generating NADPH oxidase. Two major reasons were discussed for decreases in NADPH oxidase by Ca deficiency. Superoxide generating NADPH oxidase is a membrane-bound enzyme, and any structural modification in biological membranes caused by Ca deficiency may result in a negative effect on the activity of NADPH oxidase. Secondly, in mammalian systems it has been shown that binding of Ca to acertain subunits of NADPH oxidase is essential for a proper activity (Keller et al., 1998). The role of Ca in activation of NADPH oxidase seems to be of fundamental importance for adaptive response of plants against environmental stress factors, such as pathogenic attack and for regulation of stomatal closure at extreme temperatures or drought stress (Sagi and Fluhr, 2001; Murata et al., 2001; Yang and Poovaiah, 2002). The protective role of Ca against pathogenic infection is generally ascribed to its role in cell wall structure and controlling membrane permeability (Marschner, 1995). Based on the results presented in this study together with results obtained by Sagi and Fluhr (2001), it can be suggested that Ca also protects plant cells from pathogen attack by contributing to oxidative burst via activation of $O_2^{\cdot-}$-generating NADPH oxidase.

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