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Possible roles of zinc in protecting plant cells from damage by reactive oxygen species

ISMAIL CAKMAK

Department of Soil Science and Plant Nutrition, Faculty of Agriculture, University of Cukurova, 01330 Adana, Turkey (fax +90 322 3386747; e-mail cakmak@mail.cu.edu.tr)

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Dedicated to the memory of the late Prof. Dr Drs. h. c. Horst Marschner.

CONTENTS

SUMMARY

Zinc deficiency is one of the most widespread micronutrient deficiencies in plants and causes severe reductions in crop production. There are a number of physiological impairments in Zn-deficient cells causing inhibition of the growth, differentiation and development of plants. Increasing evidence indicates that oxidative damage to critical cell compounds resulting from attack by reactive O_2 species (ROS) is the basis of disturbances in plant growth caused by Zn deficiency. Zinc interferes with membrane-bound NADPH oxidase producing ROS. In Zn-deficient plants the iron concentration increases, which potentiates the production of free radicals. The Zn nutritional status of plants influences photooxidative damage to chloroplasts, catalysed by ROS. Zinc-deficient leaves are highly light-sensitive, rapidly becoming chlorotic and necrotic when exposed to high light intensity. Zinc plays critical roles in the defence system of cells against ROS, and thus represents an excellent protective agent against the oxidation of several vital cell components such as membrane lipids and proteins, chlorophyll, SH-containing enzymes and DNA. The cysteine, histidine and glutamate or aspartate residues represent the most critical Zn-binding sites in enzymes, DNA-binding proteins (Zn-finger proteins) and membrane proteins. In addition, animal studies have shown that Zn is involved in inhibition of apoptosis (programmed cell death) which is preceded by DNA and membrane damage through reactions with ROS.

Key words: iron accumulation, membrane damage, NADPH oxidase, photooxidation, stress tolerance, superoxide dismutase, superoxide radical, zinc.

I. INTRODUCTION

Zinc deficiency is a common micronutrient deficiency in plants growing in different climatic regions of the world, particularly in arid and semi-arid regions where alkaline soils predominate (Welch *et al.*, 1991; Takkar & Walker, 1993; White & Zasoski, 1999). In an extensive soil survey, Sillanpää (1990) found approx. 50% of the soil samples collected in 25 countries to contain a deficient level of plant-available Zn. Consequently, severe depressions in plant growth and yield have been reported for many countries, for example Australia (Graham *et al.*, 1992), Turkey (Cakmak *et al.*, 1996a, 1999) and India (Takkar *et al.*, 1989).

Several physiological processes are impaired in plants suffering from Zn deficiency. Zinc deficiency causes rapid inhibition of plant growth and development, and thus of final yield. Zinc plays a fundamental role in several critical cellular functions such as protein metabolism, gene expression, structural and functional integrity of biomembranes, photosynthetic C metabolism and IAA metabolism (Marschner, 1995). Compared with other micronutrients, Zn exists in biological systems in high concentrations, particularly in biomembranes. According to Williams (1988), concentrations of Zn in animal systems range from $<10^{-9}$ M in cytoplasm to $>10^{-3}$ M in some membrane vesicles. There are many binding sites for Zn within the membranes, particularly in the interior sites of membranes. As shown with brush border membranes, the maximal binding capacity of membranes is around 400 nmol Zn^{2+} per mg protein (Prasad *et al.*, 1996). In roots of wheat seedlings, cytoplasmic concentration of total Zn has been estimated at approx. 0.4 mM (Santa Maria & Cogliatti, 1988). Most of the critical functions of Zn in cells are related to its ability to form tetrahedral coordination bonds in different vital cell constituents. Cysteine, histidine and aspartate or glutamate are major cellular ligands of Zn that form tetrahedral coordinations (Williams, 1988; Vallee & Auld, 1990; Vallee & Falchuk, 1993). These ligands, especially cysteine and histidine, bind to Zn with a greater affinity and with more stability than to Fe (Berg & Shi, 1996). Thus free radical formation, via reactions between Fe and cysteine and histidine residues, is blocked in the presence of adequate Zn (Searle & Tomasi, 1982; Girotti *et al.*, 1985; Bray & Bettger, 1990).

An increase in the level of reactive O_2 species (ROS) and a decrease in detoxification mechanisms may be the major reasons for impairment of various cellular functions in Zn-deficient plants. Zinc is required for detoxification of ROS including $O_2^{\bullet-}$ (superoxide radical) and H_2O_2 (hydrogen peroxide). Because of its fundamental role in the activation and expression of genes (Klug & Rhodes, 1987; Vallee & Falchuk, 1993), Zn might be involved in oxidative stress-induced expression of genes encoding antioxidative defence enzymes such as H_2O_2-scavenging ascorbate peroxidase and glutathione reductase (Gressel & Galun, 1994; Allen, 1995; Alscher *et al.*, 1997). Several studies have reported that low amounts of Zn in plant cells may enhance production of $O_2^{\bullet-}$ during photosynthetic electron transport (Marschner & Cakmak, 1989; Cakmak *et al.*, 1995; Cakmak & Engels, 1999) and induce $O_2^{\bullet-}$-generating membrane-bound NADPH oxidase (Cakmak & Marschner, 1988a; Pinton *et al.*, 1994).

Evidence from animal studies has shown that Zn interacts with the binding of Fe to membranes and thus restricts Fe-induced production of highly toxic hydroxyl radicals ($OH^{\bullet}$), a reaction which is referred to as site-specific $OH^{\bullet}$ production (Girotti *et al.*, 1985; Powell *et al.*, 1994). Also, apoptosis (programmed cell death) is initiated by ROS (Greenberg *et al.*, 1994; Sarafian & Bredesen, 1994; Dangl *et al.*, 1996), and is stimulated by low concentrations of soluble Zn in the cytosol (Treves *et al.*, 1994; Zalewski *et al.*, 1994). These results imply that an adequate Zn supply is critically important for protection of cells against damaging attacks by ROS.

This review emphasizes the roles played by Zn in the generation and detoxification of ROS and in the protection and maintenance of cellular integrity in plants. Additionally, attention is given to animal studies dealing with the inhibitory effects of Zn on the production of ROS in animal cells. These subjects have been reviewed briefly by Brown *et al.* (1993) and Marschner (1995). For more detailed information on the role of Zn in the protection of cellular components from harmful reactions with toxic O_2 species in animal cells, readers are referred to the excellent reviews by Willson (1988) and Bray & Bettger (1990).

II. EFFECT OF ZINC ON PRODUCTION OF REACTIVE OXYGEN SPECIES

1. *Superoxide-generating NADPH oxidase*

Production of the superoxide radical ($O_2^{\bullet-}$) in plant cells is an unavoidable process that is catalysed by a one-electron reduction of molecular O_2 (Elstner, 1982; Asada & Takahashi, 1987; Asada, 1994). Further reactions of $O_2^{\bullet-}$ are responsible for the generation of even more toxic O_2 species such as the hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$). Production of $O_2^{\bullet-}$ and its derivatives can be induced when plants are exposed to various biotic and abiotic stresses. In most cases, induced production of $O_2^{\bullet-}$ is catalysed by NAD(P)H-oxidizing enzyme systems localized in different cell compartments, such as cell walls (Gross *et al.*, 1977; Cakmak *et al.*, 1987); plasma membranes (Pinton *et al.*, 1994; Doke *et al.*, 1996; Murphy & Auh, 1996); cytosol and micro-

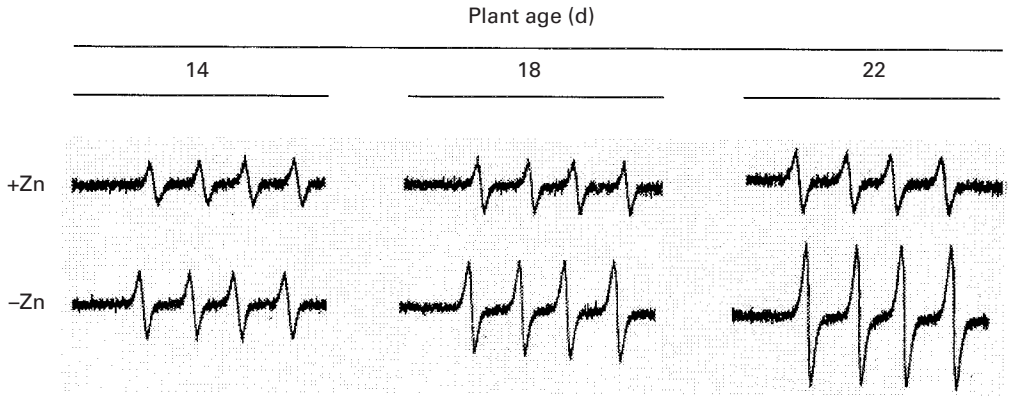


Fig. 1. Changes with plant age in the levels of $O_2^{\bullet-}$ in microsomal membrane fraction isolated from roots of cotton plants grown with (+Zn, 10^{-6} M $ZnSO_4$) and without (−Zn) Zn supply in nutrient solution. Levels of $O_2^{\bullet-}$ were determined by electron spin resonance (ESR) spectroscopy measurement of the 1,2-dihydroxybenzene-3,5-disulphonic acid (Tiron) semiquinone radical which is formed from the oxidation of Tiron by $O_2^{\bullet-}$ (McRae *et al.*, 1982). Increases in amplitude of the Tiron ESR signal indicate greater production of $O_2^{\bullet-}$ (Cakmak, 1988; Cakmak & Marschner, 1988a).

somes (Cakmak & Marschner, 1988a,b); peroxisomes (Del Rio *et al.*, 1998; Lopez-Huertas *et al.*, 1999); and mitochondria (I. Cakmak, unpublished). The production of $O_2^{\bullet-}$ and its derivatives from NAD(P)H oxidases is associated with different cellular functions, for example lignification of cell walls (Gross *et al.*, 1977), resistance against pathogen infection (Mehdy *et al.*, 1996; Bolwell & Wojtaszek, 1997), and reduction and uptake of iron by roots (Cakmak *et al.*, 1987).

Toxic O_2 species produced by NADPH-dependent oxidases are also involved in damage to several critical cell constituents (Svingen *et al.*, 1979; Jabs *et al.*, 1996; Wojtaszek, 1997). Many enzymatic and non-enzymatic lipid peroxidation processes show a high dependency on NADPH as a basic electron donor, particularly in the presence of redox active transition metals such as Fe. Supplementation of NADPH, together with ferric iron, to isolated cell compartments from animal cells (such as microsomes and mitochondria) results in a rapid peroxidation of lipids (Minotti, 1990; Glinn *et al.*, 1991). Moreover, the role of soybean lipoxygenase in oxidative cell stress is possibly related to the ability of this enzyme to produce $O_2^{\bullet-}$ by oxidizing NADPH (Roy *et al.*, 1994).

The activity of $O_2^{\bullet-}$-generating NADPH oxidases is greatly influenced by Zn deficiency in animal and plant cells. Zinc exerts a strong inhibitory effect on the generation of $O_2^{\bullet-}$ by NADPH oxidase. In Zn-deficient animal cells, NADPH-dependent production of $O_2^{\bullet-}$ was much higher than in Zn-sufficient cells, and this was considered as a major reason for Zn deficiency-induced membrane damage (Chvapil, 1979; Hammermüller *et al.*, 1987). Hammermüller *et al.* (1987) reported that Zn deficiency results in a two- to three-fold increase in NADPH-dependent H_2O_2 production in rat microsomes. However, under the same conditions Cu deficiency did not affect NADPH-dependent H_2O_2 production. In agreement with these results, Burke & Fenton (1985) reported a 10-fold increase in NADPH-dependent lipid peroxidation in Zn-deficient rat cells. Similarly, in plant cells Zn interferes with NADPH-dependent $O_2^{\bullet-}$ generation. In microsomal membrane and cytosolic fractions isolated from cotton roots, levels of $O_2^{\bullet-}$ measured by electron spin resonance (ESR) spectroscopy were higher in Zn-deficient than in Zn-sufficient plants. Levels of $O_2^{\bullet-}$ obtained by ESR spectroscopy are determined using Tiron as a spin probe for $O_2^{\bullet-}$ (McRae *et al.*, 1982). The amplitude of the Tiron–ESR signal in the microsomal membrane fraction from cotton roots is shown in Fig. 1, and reveals a progressive increase in signal with the severity of Zn deficiency, but the signal was not affected in Zn-sufficient plants. The changes in amount of $O_2^{\bullet-}$ measured directly by ESR spectroscopy were positively correlated with the activity of an $O_2^{\bullet-}$-generating NADPH oxidase in the same microsomal membranes and cytosolic fractions (Cakmak & Marschner, 1988a,b). This result indicated a role of $O_2^{\bullet-}$-generating NADPH oxidase in the level of $O_2^{\bullet-}$ measured by ESR spectroscopy. Activity of $O_2^{\bullet-}$-generating NADPH oxidase in Zn-deficient roots is very sensitive to Zn; resupply of Zn to Zn-deficient plants rapidly decreased the generation of $O_2^{\bullet-}$ in microsomal membranes (Cakmak & Marschner, 1988b). The activity of $O_2^{\bullet-}$-generating NADPH oxidase determined in Zn-deficient plants was unstable and highly sensitive to heating or storage on ice, and had an optimum pH of 7.6 (Cakmak *et al.*, 1988a). As in the $O_2^{\bullet-}$-generating NADPH oxidases described in different animal and plant cells (Bolwell & Wojtaszek, 1997), NADPH oxidase in Zn-deficient root cells also shows a high dependency on

Table 1. *NAD(P)H oxidation and $O_2^{\bullet-}$ production in plasma membrane vesicles isolated from roots of bean plants grown without (−Zn) and with (+Zn = 2×10^{-6} M) Zn supply*

	+Zn	−Zn	±Zn
	(nmol mg^{-1} protein min^{-1})		
NAD(P)H oxidation			
NADH	3.3 (100)	8.4 (264)	6.4 (194)
NADPH	2.5 (100)	6.6 (264)	4.3 (172)
$O_2^{\bullet-}$ production			
NADH	4.3 (100)	9.1 (212)	6.9 (160)
NADPH	2.8 (100)	8.2 (293)	4.8 (171)

±Zn indicates re-supply of 4×10^{-6} M Zn to deficient plants 24 h before vesicle isolation. Data are means of three independent preparations of membrane vesicles. Standard deviation did not exceed 5% of the means. Values in parentheses indicate activities presented as a percentage of those in Zn sufficient (+Zn) plants (Pinton *et al.*, 1994).

a flavin (as an electron carrier) for a maximal activity (Cakmak & Marschner, 1988a; Pinton *et al.*, 1994).

Based on these results, it is possible that Zn-deficient cells have enhanced activity of a plasma membrane-bound $O_2^{\bullet-}$-generating NADPH oxidase. This assumption was tested using isolated plasma membrane vesicles from roots of Zn-deficient and Zn-sufficient bean plants (Pinton *et al.*, 1994). As with the results obtained with microsomal membrane fractions, the activity of NADPH-dependent $O_2^{\bullet-}$-generating oxidase was strongly increased by Zn deficiency in plasma membrane vesicles (Table 1). Pinton *et al.* (1994) also showed that Zn deficiency-induced $O_{2.-}$ generation was greater when NADPH instead of NADH was used as electron donor (Table 1). The $O_2^{\bullet-}$-generating NADPH oxidase activity of Zn-deficient membranes is very similar to that induced in plant cells or animal phagocytotic cells in response to pathogenic infection (Doke *et al.*, 1996; Bolwell & Wojtaszek, 1997). Increases in plasma membrane-bound NADPH oxidase activity as a result of pathogen invasion are associated with high production of $O_2^{\bullet-}$ and other harmful O_2 species, and are produced in order to kill the pathogens and activate defence-related genes in host cells (Mehdy *et al.*, 1996). It is not known if Zn interferes with pathogen-induced NADPH-dependent $O_2^{\bullet-}$ generation in plant cells. In animal systems, $O_2^{\bullet-}$-generating NADPH oxidase of phagocytic cells was strongly decreased by high Zn concentrations (Suziki *et al.*, 1985). In contrast, magnesium was required for optimal activity (Cross *et al.*, 1999).

Enhanced production of ROS by NADPH-dependent oxidases is a common phenomenon occurring during apoptosis (Dangl *et al.*, 1996; Jabs *et al.*, 1996; Wojtaszek, 1997). Reactive O_2 species causing membrane and DNA damage have been shown to be major mediators of apoptosis (Sarafian & Bredesen, 1994; Ridgley *et al.*, 1999). There is increasing evidence in the animal literature demonstrating an important role for Zn in inhibiting apoptosis. Treves *et al.* (1994) reported that Zn is the most abundant intracellular metal and functions as a key intracellular regulator of apoptosis. A decrease in intracellular Zn^{2+} concentration triggers apoptotic cell death by inducing DNA degradation. Similarly, Zalewski *et al.* (1993, 1994) reported that the concentration of readily soluble Zn compounds in the cytosol plays a determining role in preventing DNA fragmentation, and thus apoptosis. According to Parat *et al.* (1997), inhibitory action of Zn on apoptosis seems to be related to its role in protecting cell membranes and DNA from damaging attack by toxic O_2 species. In view of the inhibitory effect of Zn on NADPH oxidase, it is logical to assume that prevention of apoptosis by Zn can be attributed to a reduced activity of NADPH oxidases involved in ROS-mediated apoptotic cell death. This area is an interesting research topic for further study. Recently, a zinc-finger protein encoded by the *Arabidopsis LSD1* gene was described as being involved in the repression of superoxide-dependent cell death in plants (Dietrich *et al.*, 1997).

The enhancing effect of Zn deficiency on $O_2^{\bullet-}$-generating NADPH oxidase can be ascribed to the dual effects of Zn on enzyme activity. First, Zn interferes with enzymatic oxidation of NADPH either by a specific binding of Zn to NADPH (Chvapil, 1979) or by an alteration of the redox properties of the NADPH-oxidizing enzyme complex (Jeffery, 1983). Accordingly, Zn could decrease the NADPH oxidation capacity of microsomal and plasma membrane fractions (Cakmak & Marschner, 1988b; Table 1). Secondly, the inhibitory effect of Zn on NADPH-dependent $O_2^{\bullet-}$-generating oxidase

can also be related to greater activity of $O_2^{\bullet-}$-scavenging superoxide dismutases (SODs) in Zn-sufficient cells (section IV).

2. *Zinc deficiency potentiates iron-mediated free radical production*

(a) *Iron accumulation in zinc-deficient plants*. In many monocotyledonous and dicotyledonous crop species grown either in soil or solution culture, Zn deficiency results in a high accumulation of Fe in roots and shoots. In a nutrient solution experiment with nine plant species, Rahimi & Bussler (1979) reported an excessive accumulation of Fe in leaves of Zn-deficient plants. Iron concentration of leaves ranged between 180 and 800 mg kg^{-1} d. wt for Zn-deficient plants, and between 75 and 300 mg kg^{-1} d. wt for Zn-sufficient plants. In a further nutrient solution study with 10 different wheat cultivars, shoot concentrations of Fe in Zn-deficient compared to Zn-sufficient plants were, on average, twofold higher at the beginning of Zn deficiency and four-fold higher under severe Zn deficiency (Cakmak *et al*., 1996b). Such high accumulation of Fe was also shown in maize and wheat plants grown on Zn-deficient soils (Warnock, 1970; I. Cakmak, unpublished). Leaves of maize plants grown in a Zn-deficient calcareous soil contained 573 mg Fe kg^{-1} d. wt, but when plants were supplied with adequate Zn the leaves contained only 80 mg Fe kg^{-1} d. wt (Warnock, 1970). Jackson *et al*. (1967) found up to 1580 mg Fe kg^{-1} d. wt in leaves of Zn-deficient maize plants grown under field conditions. Application of Zn to maize plants markedly reduced Fe concentration in leaves. Ambler *et al*. (1970) made similar observations on soybean plants growing in nutrition solution. They demonstrated a substantial decrease in Fe concentration in xylem exudate with increasing concentration of Zn from a deficient to an adequate level in nutrient solution.

Accumulation of Fe in Zn-deficient plants is particularly pronounced under high light intensity. In bean plants grown in nutrient solution, increases in light intensity from 80 to 500 μmol m^{-2} s^{-1} enhanced Fe concentration from 204 to 242 mg kg^{-1} d. wt for Zn-sufficient plants, and from 269 to 1203 mg kg^{-1} d. wt for Zn-deficient plants (Cakmak, 1988). Such dramatic changes in Fe concentration of Zn-deficient leaves were not found for Mn concentration. It is possible that Zn deficiency has a specific stimulating effect on root uptake and root-to-shoot translocation of Fe. Excessive accumulation of Fe in Zn-deficient plants found in various growth-chamber, glasshouse and field experiments could not be explained by a 'concentration effect' (i.e. by decreased biomass production) resulting from Zn deficiency-induced growth inhibition (Jackson *et al*., 1967; Warnock, 1970; Brown & Jones, 1977; Cakmak *et al*., 1996b). However in some nutrient solution experiments, Zn deficiency either did not affect or even decreased Fe uptake and translocation, although Zn-deficient plants had higher Fe concentrations in shoots (Walter *et al*., 1994; Rengel *et al*., 1998a). As discussed below, such high levels of accumulation of Fe in plant tissues can be responsible for excessive production of toxic O_2 species and extensive cellular damage.

(b) *Iron-induced production of free radicals*. High levels of accumulation of Fe in plant or animal cells are responsible for the initiation of severe oxidative stress because they produce ROS by various cellular reactions (Halliwell & Gutteridge, 1984; Price & Hendry, 1991; Hendry, 1993; Becanne *et al*., 1998). Interestingly, the role of Fe in generating ROS and causing cellular damage in Zn-deficient plants has not been studied, and has only rarely been discussed in the literature.

The powerful oxidant $OH^{\bullet}$ is produced by the Fe-catalysed Haber–Weiss reaction. This reaction involves reduction of Fe(III) by $O_2^{\bullet-}$ to Fe(II) (reaction 1), and reoxidation of Fe(II) by H_2O_2 to $OH^{\bullet}$ (reaction 2), which is known as the Fenton reaction. The requirement of Fe to cause oxidative cell damage is not only dependent on $OH^{\bullet}$ production by the Fenton reaction. There are also certain Fe species which are highly toxic and produced by the reaction of Fe^{2+} with O_2, for example, the ferryl ($Fe^{2+}O$), perferryl ($Fe^{2+}O_2$) species, or an unknown $Fe^{2+}\ldots Fe^{3+}$ complex (Girotti, 1985; Minotti, 1990; Tang *et al*., 1997). It has recently been reported that in peroxidation of membrane lipids some Fe^{2+}/O_2 species are more effective than the $OH^{\bullet}$ produced by the Fenton reaction (Qian & Buettner, 1999).

$$O_2^{\bullet-} + Fe(III) \rightarrow O_2 + Fe(II) \qquad \text{Reaction 1}$$

$$H_2O_2 + Fe(II) \rightarrow OH^{\bullet} + OH^{-} + Fe(III) \qquad \text{Reaction 2}$$

Most of the Fe in plant cells is present in the ferric form (FeIII) and it is stored in phytoferritin in leaves (Marschner, 1995). Iron is also chelated with low-molecular-weight compounds such as citrate, ADP and amino acids. Such ferric compounds can be reduced with $O_2^{\bullet-}$ (reaction 1) or several physiological reductants such as ascorbic acid. The reduced ferrous (FeII) compounds can then be oxidized, causing the production of H_2O_2 (reaction 3) or $O_2^{\bullet-}$ (reaction 4) (Halliwell & Gutteridge, 1984; Toyokuni, 1996).

$$2Fe(II) + O_2 + 2H^{+} \rightarrow 2Fe(III) + H_2O_2 \qquad \text{Reaction 3}$$

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\bullet-} \qquad \text{Reaction 4}$$

Increased Fe accumulation (such as that found for Zn-deficient plants) has also been reported for plants subjected to various stress conditions such as root

anoxia, drought and light (Price & Hendry, 1989, 1991; Hendry, 1993). Under these stress conditions accumulation of Fe is associated with enhanced lipid peroxidation and chlorophyll damage. In the case of drought stress, chloroplast membranes enhanced their production of $O_2^{\bullet -}$ in response to Fe accumulation, and Fe-catalysed formation of O_2 radicals has been considered as a major factor contributing to drought damage in plant cells (Price & Hendry, 1989, 1991).

Plants grown under flooded conditions accumulate high levels of Fe (Yamaguchi, 1989; Sahrawat *et al.*, 1996). During exposure of flooded plants to O_2, oxidation of Fe(II) leads to production of $O_2^{\bullet -}$ which is suggested as a cause of flooding damage to plants (Hendry & Brocklebank, 1985; Hendry, 1993; Neue *et al.*, 1998). Thongbai *et al.* (1999) studied the generation of free radicals in rice plants growing in an Fe-toxic soil and showed that post-anoxic injury in rice plants is related to Fe-induced production of free radicals measured by electron paramagnetic resonance spectroscopy. Such reactions would be more distinct in Zn-deficient plants because of the high accumulation of Fe in these plants under flooded conditions (e.g. in rice, Forno *et al.*, 1975; Neue *et al.*, 1998), and because of their reduced ability to defend against free radical reactions (section IV).

Iron can also stimulate free radical reactions by binding to critical cell constituents such as proteins, phospholipids and DNA. As shown in animal cells, redox cycling of Fe (also Cu) at or near the binding sites of cell constituents causes production of $OH^{\bullet}$ via the Haber–Weiss reaction (reactions 5–6). This reaction has been called site-specific $OH^{\bullet}$ production, and leads to site-specific damage in cells (Girotti, 1985; Thomas *et al.*, 1986; Chevion, 1988). As a result of its extremely short half-life and poor diffusion, the level of $OH^{\bullet}$ should be particularly high at the Fe-binding sites in cells, such as $-$SH groups and phosphate groups of cell membranes (reactions 5 and 6).

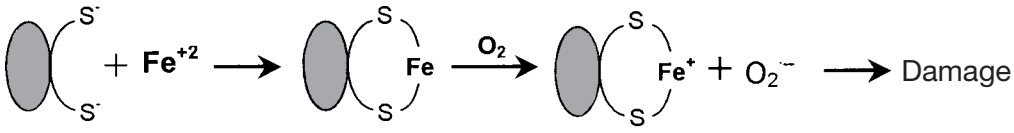
$$\text{membrane-Fe(III)} + O_2^{\bullet -} \rightarrow O_2 + \text{membrane-Fe(II)} \qquad \text{Reaction 5}$$

$$H_2O_2 + \text{membrane-Fe(II)} \rightarrow OH^{\bullet} + OH^{-} + \text{membrane-Fe(III)} \qquad \text{Reaction 6}$$

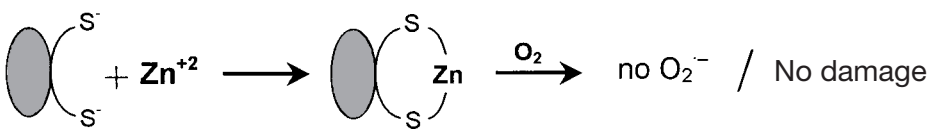
In most cases, Fe and Zn compete for the binding sites on proteins and phospholipids. The ability of Zn to compete with Fe at binding sites on the membrane has been suggested by Girotti *et al.* (1985) and Bray & Bettger (1990) as an explanation for the increased lipid peroxidation under Zn deficiency. They suggest that the existence of adequate amounts of Zn in the vicinity of Fe-binding sites blocks binding of Fe and thus inhibits Fe-catalysed site-specific generation of $OH^{\bullet}$. In support of this theory, Kunimoto *et al.* (1981) observed that membrane lipids with negative surface charges (i.e. Fe-binding sites) are more rapidly oxidized by the addition of Fe than membrane lipids carrying a positive charge. This result emphasizes the importance of Fe-binding sites of membranes in the generation of free radicals and peroxidative cell damage. Hydroxyl radicals ($OH^{\bullet}$) produced at Fe-binding sites on membranes can rapidly initiate peroxidation of phospholipids, causing site-specific damage.

Willson (1988) also suggested that protection of biomembranes and maintenance of cellular integrity by Zn are predominantly controlled by binding of Zn to SH-containing compounds, particularly in membrane proteins. As given schematically in reactions 7 and 8 (Willson, 1988), under Zn-deficient conditions membrane $-$SH groups can be occupied by Fe, with a concomitant generation of $O_2^{\bullet -}$ and membrane damage (reaction 7). By competing with Fe, or other redox active metals such as Cu(II), for binding to $-$SH groups of membrane proteins, Zn inhibits metal-induced generation of $O_2^{\bullet -}$ and the related membrane damage (Willson, 1988). When bound to $-$SH groups or other critical Fe-binding sites of membranes, Zn, as a redox-inactive element in biological systems, cannot undergo a cyclic reduction and reoxidation to produce free radicals (reaction 8).

Evidence for the cellular importance of the binding of Zn to $-$SH groups was shown *in vitro* by Searle & Tomasi (1982) in a model study. They compared Zn and Fe for their role in the production of the hydroxyl radical ($OH^{\bullet}$) in the presence of cysteine. When incubated with $FeSO_4$, cysteine (RS^{-}) complexes with Fe^{2+} (reaction 9), and this Fe^{2+}–cysteine complex is oxidized producing a ferric complex and



Reaction 7



Reaction 8

$O_2^{\bullet-}$ (reaction 10). The superoxide radical produced reacts with a new Fe^{2+}–cysteine complex to give rise to H_2O_2 (reaction 11). Hydrogen peroxide, which can also be produced by non-enzymatic dismutation of $O_2^{\bullet-}$ (reaction 12), is reduced by Fe^{2+}-cysteine complex to $OH^{\bullet}$, as in the Fenton reaction (reaction 13).

$$Fe^{2+} + 2RS^- \rightarrow Fe^{2+}(RS^-)_2 \quad \text{Reaction 9}$$

$$Fe^{2+}(RS^-)_2 + O_2 \rightarrow Fe^{3+}(RS^-)_2 + O_2^{\bullet-} \quad \text{Reaction 10}$$

$$Fe^{2+}(RS^-)_2 + O_2^{\bullet-} \rightarrow Fe^{3+}(RS^-)_2 + H_2O_2 \quad \text{Reaction 11}$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{Reaction 12}$$

$$H_2O_2 + Fe^{2+}(RS^-)_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+}(RS^-)_2 \quad \text{Reaction 13}$$

In the presence of adequate amounts of $ZnSO_4$, Zn^{2+} ions bind to cysteine, blocking binding of Fe to cysteine and thus preventing formation of $Fe^{2+}(RS^-)_2$ and ROS during the redox cycling of Fe–cysteine complex. Zinc is catalytically inert with respect to reactions that produce free radicals when bound to cysteine or other relevant ligands. According to Berg & Shi (1996), specific binding of Zn to tetrahedral sites of a large number of vital cell compounds, and the lack of redox activity of Zn, may be the bases for many of its crucial physiological functions in cells. Accordingly, it has been found that inhibition by Zn of peroxidative damage in animal cells is related to displacement of redox active metals (e.g. Fe and Cu) from cellular binding sites and, consequently, its ability to inhibit the site-specific production of ROS (Girotti *et al*., 1985; Powell *et al*., 1994).

Zinc protects DNA-binding proteins (Zn-finger proteins) from reacting with Fe. In these proteins, Zn is tetrahedrally coordinated with cysteinyl–SH groups and imidazole histininyl groups (Vallee & Auld, 1990; Rhodes & Klug, 1993). Under conditions of high-Fe and low-Zn concentrations, Zn bound to the finger protein can be exchanged by Fe(II), and Fe-substituted finger protein, along with H_2O_2, can induce generation of the potent oxidant $OH^{\bullet}$ (Conte *et al*., 1996). According to Conte *et al*. (1996), because of the very close proximity of the Zn-finger protein to DNA, Fe-substituted Zn-finger proteins may cause extensive DNA damage via $OH^{\bullet}$. DNA damage and impairments in gene expression, as described in animal cells, can also be expected in Zn-deficient plant cells containing high Fe concentrations. Further studies are needed on this effect in plants.

3. *Zinc deficiency-enhanced photooxidation*

(a) *Decrease in photosynthesis*. Chloroplasts are the major cell compartments producing $O_2^{\bullet-}$ and $O_2^{\bullet-}$-derived toxic O_2 species such as $^1O_2$ and $OH^{\bullet}$ (Asada & Takahashi, 1987; Elstner & Osswald, 1994). During photosynthetic electron transport, part of the reducing equivalents generated are transferred to molecular O_2 with the concomitant production of $O_2^{\bullet-}$ in the chloroplastic stroma, which is called the Mehler reaction. It is estimated that under non-stressed conditions up to 20–25% of total non-cyclic photosynthetic electron transport is consumed by the Mehler reaction at light saturation (Robinson, 1988; Osmond & Grace, 1995; Lovelock & Winter, 1996). Production of $O_2^{\bullet-}$ during photosynthetic electron transport can be intensified when plants are exposed to high light intensity combined with an environmental stress factor that restricts photosynthetic CO_2 fixation, such as chilling, drought stress and mineral nutrient deficiencies (Asada *et al*., 1977; Elstner *et al*., 1988; Cakmak & Marschner, 1992; Osmond & Grace, 1995; Biehler & Fock, 1996; Polle, 1996; Fryer *et al*., 1998; Heiser *et al*., 1998; Cakmak & Engels, 1999). Such conditions lead to limited $NADP^+$ availability for acceptance of electrons from photosystem I, thus intensifying electron flow to O_2 from ferrodoxin with a concomitant photooxidative damage to thylakoid constituents by ROS. Under these conditions, conversion of the absorbed light energy into chemical energy can be impaired, causing the transfer of excess energy from the excited triplet chlorophyll state to ground state O_2 to form singlet oxygen ($^1O_2$) (Elstner, 1982; Demming-Adams & Adams, 1992). Singlet oxygen produced in thylakoid membranes is directly responsible for the damage to proteins of the photosystem II reaction centres, especially D1 polypeptide (Hideg *et al*. 1994; Huner *et al*., 1998).

Photooxidative damage in Zn-deficient leaves can be expected as a result of impaired photosynthetic CO_2 fixation and reduced activity of superoxide dismutase (SOD). In cauliflower, reduction in photosynthesis induced by Zn deficiency is associated with a decrease in intercellular CO_2 concentration and stomatal conductance (Sharma *et al*., 1994). Stomatal limitation is considered an important reason for reduced photosynthesis in Zn-deficient plants. Sharma *et al*. (1995) reported a significant role of Zn in the regulation of the stomatal aperture. This role of Zn was ascribed to maintenance of a high K concentration in guard cells. A decrease in carbonic anhydrase activity due to Zn deficiency is well known, and may be a factor contributing to reduced photosynthesis (Ohki, 1976; Rengel, 1995a; Cakmak & Engels, 1999). Using two wheat cultivars differing in sensitivity to Zn deficiency, Fischer *et al*. (1997) showed that for both Zn-deficient and Zn-sufficient conditions, a Zn-deficiency-resistant cultivar exhibited a higher rate of net photosynthesis than a sensitive cultivar. The higher level of photosynthesis in Zn-deficiency-resistant cultivars was related to higher carbonic

anhydrase activity, because irrespective of Zn supply the resistant cultivar had an inherently higher carbonic anhydrase activity than the sensitive cultivar (Rengel, 1995a).

Inhibition of photosynthesis in Zn-deficient plants can also be a consequence of a Zn-deficiency-induced reduction in phloem sap sink demand. Severe inhibition of meristem growth is a typical response of plants to Zn deficiency, which is possibly caused by reduced levels of IAA and protein synthesis (Kitagishi *et al.*, 1987; Cakmak *et al.*, 1989). Additionally, in Zn-deficient plants there is an enhanced accumulation of carbohydrates, especially in phloem sap source leaves, possibly resulting from either impaired phloem export of carbohydrates or decreased sink demand (Sharma *et al.*, 1982; Marschner & Cakmak, 1989; Marschner *et al.*, 1996). Inhibition of photosynthesis by accumulation of carbohydrates in leaves has been well documented (Krapp *et al.*, 1991) and may be a core problem in Zn-deficiency-dependent decreases in photosynthetic CO_2 fixation.

(b) *Light-induced leaf chlorosis*. Irrespective of the underlying causes, impairments in photosynthetic CO_2 fixation in Zn-deficient plants may cause a corresponding impairment in utilization of electrons and absorbed light energy for CO_2 fixation. These conditions in Zn-deficient plants may accentuate photogeneration of ROS and photooxidative damage to chloroplasts, as shown schematically in Fig. 2.

Photooxidative damage of chloroplast pigments is particularly pronounced in leaves showing impaired phloem sap sucrose export from source to sink tissues. Inhibition of sucrose phloem transport as a result of pathogen infection of sieve tubes (Osswald & Elstner, 1986); Mg or K deficiency (Cakmak, 1994; Cakmak *et al.*, 1994); petiole phloem destruction (Wood *et al.*, 1986); or high apoplastic invertase activity (Von Schaewen *et al.*, 1990) is associated with a rapid development of leaf chlorosis, particularly under high light intensity (Dickinson *et al.*, 1991; Cakmak & Marschner, 1992). Similarly, enhancements in chlorosis and necrosis due to increased light intensity are very typical in Zn-deficient source leaves, reflected in a massive accumulation of sucrose and starch (Marschner & Cakmak, 1989) causing a high potential for photooxidative damage of chloroplast constituents (Fig. 2). In accordance with this suggestion, enhancements in light intensity from 80 to 490 μmol m^{-2} s^{-1} markedly depressed shoot elongation and stimulated appearance of leaf chlorosis under Zn deficiency, but not at adequate Zn supply (Cakmak, 1988; Fig. 3a,b). Also, partial shading of Zn-deficient leaves prevented or strongly delayed appearance of chlorosis in the shaded areas (Fig. 3c). Similar results have also been shown for citrus (Cakmak *et al.*, 1995) and rice plants (Obata *et al.*, 1997). In citrus orchards in California, Zn deficiency chlorosis has been reported to be more severe on branches directly exposed to sunlight (Chapman, 1966). Similarly, in citrus orchards in southern Turkey we observed that under Zn-deficient conditions, Zn deficiency symptoms are very slight in the north-facing parts, while south-facing parts of trees show very severe Zn deficiency chlorosis (Cakmak *et al.*, 1995; Fig. 3d). The enhancements in severity of chlorosis and necrosis under high light intensity were not caused by lower Zn concentrations in leaves. Shaded (green) and non-shaded (chlorotic/necrotic) parts of leaves had about the same Zn concentration (Marschner & Cakmak, 1989; Cakmak *et al.*, 1995). These observations, and results with light-induced chlorosis under Zn deficiency, indicate that increased severity of leaf chlorosis under high light intensity in Zn-deficient conditions is a consequence of photooxidative damage to chloroplast pigments catalysed by ROS. Similarly, in Mg- or K-deficient plants, increases in light intensity markedly stimulated the development of leaf chlorosis and necrosis. The increase in leaf chlorosis under Mg or K deficiency was associated with severe inhibition of phloem transport of sucrose from source leaves, and thus a massive accumulation of carbohydrates in leaves (Marschner & Cakmak, 1989; Cakmak & Marschner, 1992; Cakmak *et al.*, 1994). With the severity of leaf chlorosis in Mg- or K-deficient plants, activities of $O_2^{\bullet -}$- and H_2O_2-scavenging enzymes showed a substantial increase, indicating enhanced production of ROS in chloroplasts (Cakmak & Marschner, 1992; Cakmak, 1994). Such distinct effects of high light levels on development of leaf chlorosis are very specific for Zn-, Mg- and K-deficient plants, and do not occur in P-deficient plants where activities of antioxidative enzymes are not enhanced, sucrose export is not affected, and carbohydrates do not accumulate in leaves despite severe decreases in shoot growth (Cakmak, 1994; Cakmak *et al.*, 1994).

Photooxidative damage of the chloroplast constituents under Zn deficiency can also be aggravated by reduced activity of enzymes scavenging $O_2^{\bullet -}$ and H_2O_2 in chloroplasts (section IV). As the photooxidative damage is typical for plants exposed to drought, chilling and airborne pollutants such as ozone and SO_2 (Asada, 1994; Foyer *et al.*, 1994; Alscher *et al.*, 1997), the Zn nutritional status of plants under such conditions should become more important. Therefore improvement of the Zn nutritional status of plants can be of crucial importance in protecting plants from such environmental stresses (section V).

(c) *Decrease in indole-3-acetic acid*. In addition to leaf chlorosis and necrosis, decrease in leaf and fruit size and inhibition of shoot elongation are further characteristic morphological changes occurring in Zn-deficient plants. It is widely accepted that these

morphological changes in Zn-deficient plants are attributable to decreased levels of the growth hormone indole-3-acetic acid (IAA) (Skoog, 1940; Marschner, 1995). Zinc-deficiency-dependent decrease in the concentration of IAA (Cakmak *et al.*, 1989; Domingo *et al.*, 1990) is more distinct when plants are grown under higher light intensity (Skoog, 1940; Cakmak, 1988; Römheld & Marschner, 1991). In *in vitro* studies, light-induced oxidative degradation of IAA has been shown, especially in the presence of high Fe (Cakmak, 1988; Dunlop & Robacker, 1988). Iron, by catalaysing the Haber–Weiss reaction, is responsible for the production $OH^{\bullet}$. IAA is extremely sensitive to $OH^{\bullet}$, and can be oxidized rapidly upon exposure to high concentrations of $OH^{\bullet}$ (Brennan & Frenkel, 1983; Cakmak, 1988; Dunlop & Robacker, 1988). Besides non-enzymic oxidation, IAA is also oxidized by H_2O_2-dependent peroxidases (Schneider & Wightman, 1975; Gazaryan *et al.*, 1996), and $O_2^{\bullet -}$ and $^1O_2$ are involved in this degradation pathway (Metodiewa *et al.*, 1992). Higher peroxidase activity, $O_2^{\bullet -}$ and Fe in Zn-deficient plants may result in enhanced oxidative degradation of IAA. This could be another relevant reason why Zn-deficient plants become very sensitive to ROS when grown under high-light conditions.

III. MEMBRANE DAMAGE BY REACTIVE OXYGEN SPECIES

1. *Impairments in membrane structure*

One of the well established cellular functions of Zn is its role in maintaining the structural integrity and controlling permeability of biomembranes. In Zn-deficient plant cells, loss of membrane integrity and increased membrane permeability to inorganic ions were first demonstrated by Welch *et al.* (1982). In experiments with wheat, they showed that Zn deficiency caused greater leakage of ^{32}P and ^{36}Cl from root cells than in Zn-sufficient plants. Increased leakage of ions was also found in plants with adequate levels of Zn in their roots and shoots when grown in an external medium without Zn supply. In view of these results, Welch *et al.* (1982) concluded that Zn has a direct effect on the structural integrity of biomembranes. To fulfil this action, Zn must be continuously present in the external medium during plant growth. Similar observations were also made in animal studies. According to Lindsay *et al.* (1989), an increase in K leakage from Zn-deficient cells can be ascribed to the protective role of Zn at the apoplasmic side of the plasma membrane. Apparently, Zn reacts with negatively charged molecules associated with the plasma membrane, stabilizing the membrane structure (Lindsay *et al.*, 1989).

In studies with cotton, wheat, tomato and apple plants, Cakmak & Marschner (1988c) showed that leakage not only of inorganic ions, but also of organic compounds from roots, increased in response to Zn deficiency. As shown in Table 2, Zn deficiency was associated with a marked increase in leakage of amino acids, carbohydrates and phenolics from root cells. These increases in membrane permeability were rapidly reversed by a resupply of Zn to Zn-deficient plants (Cakmak & Marschner, 1988c; Zhang *et al.*, 1991). The role of Zn in membrane integrity is thought to be independent of the role of Ca, because increases in membrane permeability caused by Zn deficiency could not be reduced by a supply of Ca to the external medium (Cakmak & Marschner, 1988c). The independent roles of Ca and Zn in membrane integrity were confirmed in a model study with phospholipid vesicles by Kaszuba & Hunt (1990). They showed that both Zn and Ca are

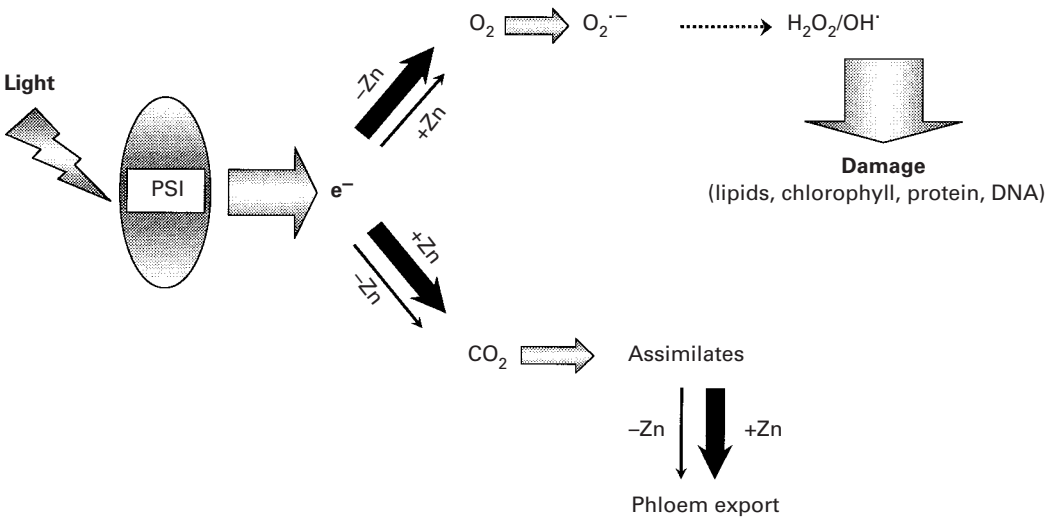


Fig. 2. Schematic representation of the photosynthetic electron flow to CO_2 and O_2 and formation of toxic oxygen species in chloroplastic stroma. +Zn and −Zn refer to adequate and deficient Zn supply, respectively.

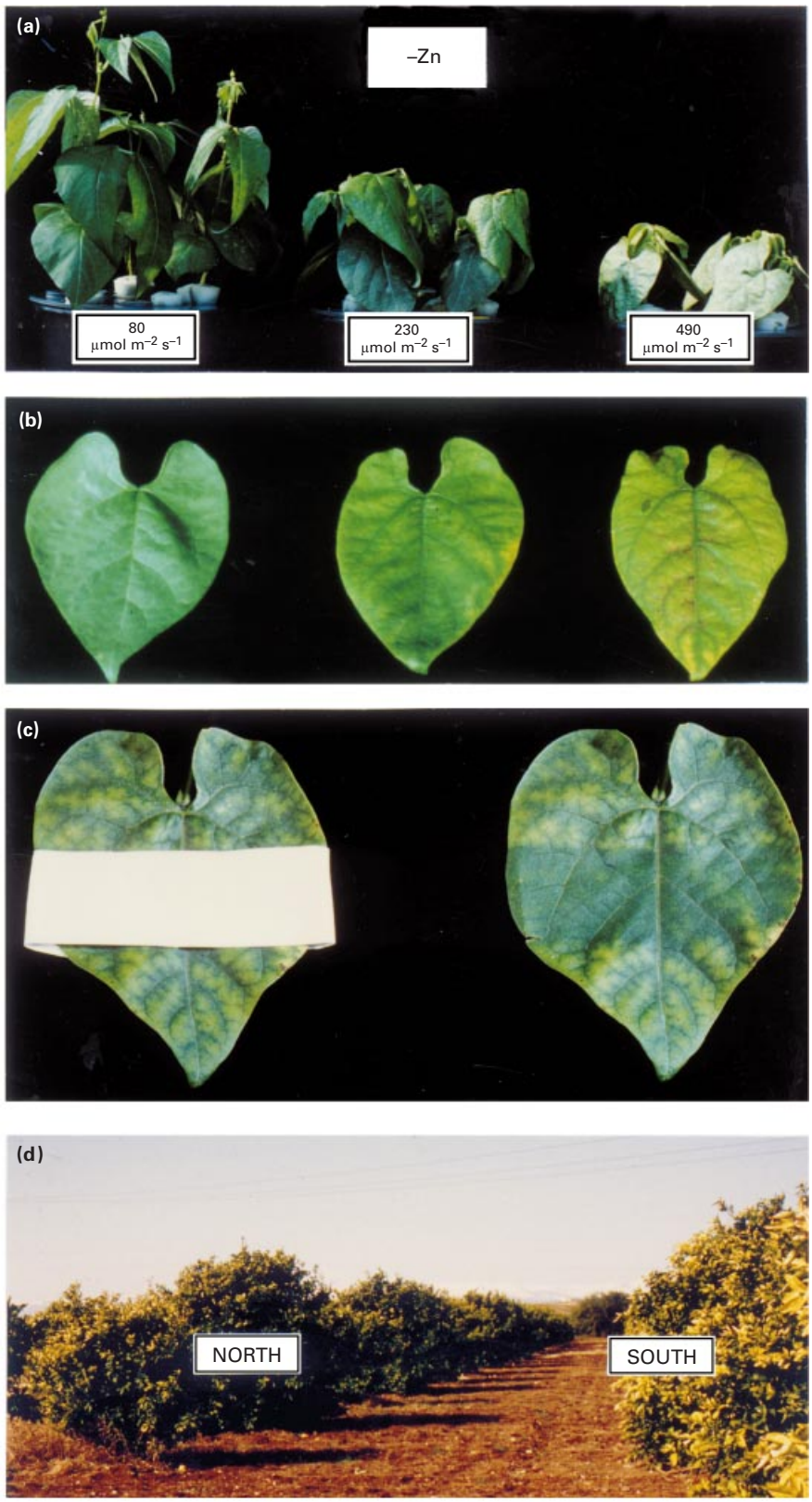


Fig. 3. (a) Effect of different light intensities on shoot growth of 20-d-old bean (*Phaseolus vulgaris*) plants in nutrient solution without Zn supply. (b) Primary leaves of 18-d-old Zn-deficient bean plants grown at light intensities of 80 (left), 230 (middle) and 490 (right) μmol m^{-2} s^{-1}. (c) Effect of partial shading on development of interveinal chlorosis in Zn-deficient primary leaf of 20-d-old bean plants. Partial shading of the leaf was started before the appearance of visual symptoms of chlorosis, and continued for 9 d. (d) Direction effect on Zn deficiency chlorosis in grapefruit trees grown at the field station of the University of Cukurova, Turkey. Zinc deficiency symptoms were severe on sun-exposed sides of trees (south-facing parts) and absent or slight in the north- and north-west-facing parts of trees. Zinc concentrations in leaves from different directions were similarly very low, ranging from 7 to 9 mg kg^{-1} d. wt.

independently required for stabilization of vesicles, as these ions bind to different sites on the membrane.

Enhanced leakage of organic compounds and nutrients from roots of Zn-deficient plants may have some ecological implications such as nutrient mobilization and infection of roots by pathogenic and non-pathogenic microorganisms (Marschner, 1995; Rengel *et al*., 1998b). Organic compounds released from roots can change the biological and chemical activity of the rhizosphere, thereby changing the availability to plant roots of micronutrients such as Zn, Cu, Ni and Mn (Zhang *et al*., 1991; Welch, 1995; Rengel *et al*., 1996; Rengel, 1997).

2. *Phospholipids and −SH groups*

Increases in membrane permeability are normally associated with peroxidation of membrane constituents, especially unsaturated fatty acids (Demopoulos, 1973; Dhindsa *et al*., 1981; Girotti, 1985). In cotton roots, reducing the Zn supply reduced levels of fatty acids, particularly unsaturated fatty acids (Table 3). Compared to Zn-adequate cotton roots, saturated fatty acids were decreased in Zn-deficient roots by 22%, and unsaturated fatty acids by 31%. The total amount of phospholipids in roots of Zn-deficient plants showed a similar decrease (Table 3). Unsaturated fatty acids are known to be more susceptible to peroxidation than saturated fatty acids because they contain double bonds (Demopoulos, 1973; Girotti, 1985). Peroxidative damage to biomembranes begins with oxidation of unsaturated bonds in fatty acids and proceeds through a series of free radical reactions, resulting in substantial alteration in membrane structure. Disturbances in the membrane architecture by peroxidative damage impair the integrity and functions of the proteins and other membrane-associated structures, causing membranes to become leaky and unstable (Demopoulos, 1973; Van Ginkel & Sevanian, 1994).

As with unsaturated fatty acids, sulfhydryl (−SH) groups in cell membranes are very sensitive to Zn deficiency. The number of −SH groups in the plasma membrane proteins of root cells was decreased by Zn deficiency in barley (Welch & Norvell, 1993) and in wheat, especially in Zn-deficiency-sensitive wheat cultivars (Table 4). Resupply of Zn to Zn-deficient wheat plants for 24 h clearly increased the number of −SH groups (Rengel, 1995b). This effect of resupplied Zn on −SH groups was ascribed to an increase in SOD activity and a decrease in $O_2^{\bullet-}$-generating NADPH oxidase in Zn-deficient roots. It could also result from Zn protection of −SH groups in membrane proteins from redox reactions with redox-active transition metals such as Fe(III) and Cu(II). Zinc also protects −SH groups in animal cell membranes. Xia *et al*. (1999) have reported that deficiency of Zn in erythrocyte membranes is associated with important decreases in the levels of −SH groups, and these decreases could be rapidly reversed by a resupply of Zn to Zn-deficient membranes. By affecting the redox status of −SH groups, Zn can exert a significant role in ion-channel gating phenomena or in the activity of transporter proteins in the plasma membranes of root cells, thereby altering ion uptake and efflux rates (Kochian, 1993; Welch & Norvell, 1993; Welch, 1995).

3. *Alterations in ion absorption*

(a) *Membrane-bound ATPases*. Impairments in the integrity of cell membranes of Zn-deficient plants can cause alterations in the activity of membrane-bound proton-pumping enzymes and ion channels, and thus affect the uptake of nutrients across the plasma membranes. Limited data exist in the literature concerning the role(s) of Zn in membrane-bound ATPase activity. In plasma membrane-enriched vesicles isolated from bean roots, Zn

Table 2. *Concentrations of amino acids, sugars and phenolics in the root exudates of cotton, wheat and apple plants grown with different Zn supplies*

Zn supply (M)		Amino acids	Sugars	Phenolics
		(μg g^{-1} d. wt root per 6 h)		
Cotton				
8×10^{-7}	(adequate)	48 ± 11	375 ± 41	117 ± 22
0	(deficient)	165 ± 16	751 ± 273	161 ± 31
Wheat				
8×10^{-7}	(adequate)	21 ± 2	315 ± 72	34 ± 6
0	(deficient)	48 ± 3	615 ± 61	80 ± 6
Apple				
5×10^{-7}	(adequate)	12 ± 3	275 ± 13	103 ± 38
0	(deficient)	55 ± 13	823 ± 36	350 ± 121

Data represent means ± SD of 4 independent replications (Cakmak & Marschner, 1988c).

Table 3. *Concentrations of fatty acids and total phospholipids in 20-d-old cotton roots supplied with different Zn concentrations*

	Zn treatment		
	Zn_0	Zn_1	Zn_2
	($\mu g\ g^{-1}$ root f. wt)		
Fatty acids			
Total	1123 ± 48(73)	1335 ± 151(87)	1531 ± 50
Saturated	532 ± 26(73)	613 ± 44(91)	677 ± 44
Unsaturated	591 ± 62(69)	721 ± 114(84)	854 ± 55
Total phospholipids	1530 ± 60(69)	1700 ± 240(76)	2230 ± 111

Zn_2, 8×10^{-7} M, adequate Zn supply; Zn_1, 2×10^{-1} M; Zn_0, 0. Values in parentheses are percentage of Zn_2 treatment. Data represent means ± SD of 3 independent replications (Cakmak & Marschner, 1988c).

Table 4. *Concentrations of reactive sulfhydryl groups in the root-cell plasma membranes of a Zn deficiency-sensitive durum wheat (cv. Durati) and Zn deficiency-resistant bread wheat (cv. Warigal) grown with sufficient (+Zn) and deficient (−Zn) Zn supply in nutrient solution*

	Zn supply		
Cultivar	Deficient	Sufficient	Mean
	(μmol −SH g^{-1} root d. wt)		
'Durati' (sensitive)	3.9	5.3	4.6
'Warigal' (resistant)	6.0	7.0	6.5
Mean	4.9	6.2	

Data represent means of 6 replications. The overall means for Zn treatments and the genotypes are given because the interaction effect was not significant ($P \leqslant 0.50$). The $HSD_{0.05}$ value is 0.7 $\mu mol\ g^{-1}$ root d. wt for both effects (Rengel, 1995b).

deficiency reduced vanadate-sensitive ATP hydrolysis and, more markedly, ATP-dependent proton-pumping activity, resulting in a decrease in pH gradient across the membrane (Pinton *et al*., 1993). A resupply of Zn to Zn-deficient plants for 24 h evidently restored the proton pumping activity of membrane vesicles (Pinton *et al*. 1993). These effects of Zn substantiate the importance of Zn in maintaining the proton gradient across membranes. Accordingly, in studies with excised maize roots it has been shown that the membrane potential of root cells is higher in the presence than in the absence of Zn, possibly because of the stimulation by Zn of plasma-membrane-bound ATPase activity (Kennedy & Gonsalves, 1987). These authors suggested that Zn^{2+}-ATP might be a substrate for plasma membrane ATPase, and higher membrane potential maintained by the presence of Zn benefits the uptake of nutrients. In a further study with the plasma membrane vesicles isolated from corn roots, Kastrup *et al*. (1996) showed that Zn in the form of $ZnATP^{2-}$, but not free Zn^{2+} activated H^+-ATPase and demonstrated the importance of Zn in regulating proton-pumping activity of plasma membranes and nutrient uptake by plant roots. Decreases in plasma-membrane-bound ATPase activity due to Zn deficiency were also found in chickpea roots, and were attributed to a modification of the enzyme complex resulting from insufficient membrane Zn (Lawrence *et al*., 1995).

Disturbances in the activity of membrane-bound ATPases caused by Zn deficiency may be a consequence of free radical attack on enzymes. For example, Na^+/K^+-ATPase and Ca-ATPase activities in animal cells are very sensitive to oxidative damage by ROS. Oxidation of −SH groups of enzymes appears to be the major reason for loss of enzyme activity in stressed cells. In studies with animal cells, Kurella *et al*. (1995) reported the existence of a relationship between the decrease in Na^+/K^+-ATPase activity and oxidation of −SH groups in the enzyme by free radicals. Oxidation of one or two −SH groups per mole enzyme resulted in a 43% decrease in enzyme activity, and with oxidation of an additional three to four −SH groups per mole enzyme there was a decrease of 70% in enzyme activity. Similarly, in plant cells oxidation of ATPase −SH groups in purified plasma membrane vesicles

from *Elodea canadensis* resulted in a significant loss of ATPase activity (Elzenga *et al.*, 1989).

(b) *Nutrient uptake*. Impaired activity of the ATP-dependent proton gradient across the plasma membrane may result in changes in ion absorption by root cells. Among the mineral nutrients, uptake of P was especially influenced by Zn deficiency. In a number of plant species, Zn deficiency is associated with a marked increase in P uptake by roots (Loneragan *et al.*, 1982; Cakmak & Marschner, 1986; Rengel & Graham, 1995; Parker, 1997). As a consequence, symptoms of Zn deficiency can be mistaken for symptoms of P toxicity. In contrast to P, net uptake of NO_3^- was substantially depressed by Zn deficiency (Marschner & Cakmak, 1986; Cakmak & Marschner, 1990; Norvell & Welch, 1993). Decreases in net NO_3^- uptake are attributed to enhanced net efflux of NO_3^- through the plasma membranes, due to its enhanced membrane permeability under Zn deficiency (Cakmak & Marschner, 1990). Zinc deficiency also alters the uptake of other nutrient elements such as Fe (section II, 2a), B, Mg, K, Mn and Cl, but not consistently (Marschner & Cakmak, 1986; Graham *et al.*, 1987; Norvell & Welch, 1993; Parker, 1997). As shown for uptake of micronutrients and P in different wheat genotypes, the effects of Zn deficiency on uptake of mineral nutrients can be genotype-dependent (Rengel & Graham, 1995).

Zinc sufficiency is also necessary to control the uptake and accumulation of Na in plants (Shukla & Prasad, 1974; Norvell & Welch, 1993) and in algae (Rygol *et al.*, 1992). Increased Na uptake under Zn deficiency is possibly related to impairments in membrane stability and thus a loss of the cell's ability to control Na uptake. Using yeast protoplasts, Kovac *et al.* (1987) showed that Zn has a particular role in maintaining membrane stability and protecting membranes against osmotic stress. In the absence of Zn, plasma membranes of yeast protoplasts lost their stability and became very permeable, leaking intercellular solutes. As suggested by Norvell & Welch (1993), improvement of the Zn nutritional status of plants growing in saline soils of arid and semi-arid regions is important not only to overcome Zn-deficiency-related decreases in plant productivity, but also to protect plants against excessive salinity and resulting injury.

(c) *Changes in activity of ion channels*. Significant progress has been made in understanding the importance of ion channels in ion transport across the membranes. Ion channels are biologically very active proteins that permit rapid and selective transport of ions depending on their opening and closure status (Tester, 1990). Ion channel proteins contain reactive −SH groups, and the activity of the channel is dependent on the redox status of these −SH groups. There is some evidence of involvement of Zn in regulating the activity of ion channels in animal cells. In frog muscle cells a low concentration of extracellular Zn caused an increase in K efflux via K channels (Spalding *et al.*, 1986). This effect of Zn was attributed to the binding of Zn to histidine residues in the K channel protein. Zinc may also interact with Cl^- channels. In *Xenopus* oocytes, extracellular Zn reduced Cl^- efflux via a Cl^- channel by facilating the slow gating of the channel (Chen, 1998). Reduction in Cl^- efflux across Cl^- channels, resulting from increasing concentration of extracellular Zn, has been explained by the binding of Zn to channel molecules in the ionic form, $ZnOH^+$ (Spalding *et al.*, 1990).

In plant and algal cells Zn exerts similar inhibitory roles on efflux of ions via anion channels (Hedrich & Kurkdjian, 1988; Tyerman, 1992). Using isolated vacuoles from sugar beet, Hedrich & Kurkdjian (1988) showed that Zn strongly reduces activity of the Ca-activated, slow vacuole-type anion channels. This blocking action of Zn on channel activity occurs in a few minutes of exposure of vacuoles to a very low Zn concentration (0.1 μM $ZnCl_2$). In wheat and barley seedlings, Welch and co-workers (Welch *et al.*, 1982; Norvell & Welch, 1993; Welch & Norvell, 1993) reported that Zn-deficient root cells enhanced leakage of Cl^- compared with Zn-sufficient plants. It seems likely that Zn may affect the activity of the putative Cl^- channels in root cell membranes by interacting with −SH groups in sulphur-containing amino acid constituents of membrane peptides.

In view of decreases in the amount of −SH groups in the root cell plasma membranes caused by Zn deficiency, Welch & Norvell (1993) suggested that Zn is required for protection of membrane −SH groups against free radicals, thus influencing activity of ion transport proteins as well as ion channels. Alterations in influx and efflux of ions in Zn-deficient plants, compared with Zn-sufficient ones, may at least in part be attributed to the redox state of reactive −SH groups contained in ion channels.

In most cases, unregulated Ca accumulation in the cytosol initiates programmed cell death (Dangl *et al.*, 1996; Levine *et al.*, 1996). Toxic O_2 species are involved in increased concentrations of cytosolic Ca^{2+} because they oxidize critical residues in Ca channel proteins. In animal cells, Ca efflux from Ca-accumulating organelles is enhanced when −SH residues in Ca channels are oxidized by toxic O_2 species (Stoyanovsky *et al.*, 1994; Donoso *et al.*, 1997). Similarly, in Zn-deficient plant cells an enhanced free radical-dependent oxidation of −SH groups and other critical residues of Ca channels might be expected. This could result in an enhanced Ca efflux into the cytosol from Ca-accumulating organelles (e.g. vacuole and endoplasmatic reticulum), thereby causing a dramatic increase in

cytosolic Ca concentration. Protection of the critical −SH groups in Ca channels by Zn has been reported in different animal cells (Chiamvimovat *et al.*, 1995, cited by Xia *et al.*, 1999). Protection of −SH groups by improved Zn nutrition may be provided by reduced activity of $O_2^{\bullet-}$-generating NADPH oxidase (section II, 1). Hypothetically, maintenance of low cytosolic Ca concentration may be impaired by Zn deficiency, triggering programmed cell death. For plant cells, there are no studies or discussion in the literature related to the death of Zn-deficient cells as a possible consequence of increased cytosolic Ca concentration or DNA degredation. This point needs to be clarified in future studies.

IV. DETOXIFICATION OF REACTIVE OXYGEN SPECIES

1. *Superoxide dismutases*

The superoxide radical and its derivates are unavoidable products of normal cell metabolism, and their generation is particularly high during electron transport in chloroplasts and mitochondria (Cakmak *et al.*, 1993; Elstner & Osswald, 1994; Polle, 1996; Alscher *et al.*, 1997). To minimize the destructive effects of ROS, plant cells are equipped with various antioxidants and antioxidative defence enzymes such as superoxide dismutases (SODs), ascorbate peroxidases, ascorbic acid, α-tocopherol and carotenoids (Asada, 1994; Cakmak, 1994; Foyer *et al.*, 1994). By catalysing detoxification of $O_2^{\bullet-}$ to O_2 and H_2O_2 and blocking $O_2^{\bullet-}$-driven cell damage, SODs are a major component of the antioxidative defence system of plant cells (Fridovich, 1986; Bowler *et al.*, 1994). According to their metal cofactor, SODs are classified into three types containing either Mn (Mn-SOD), Fe (Fe-SOD) or Cu and Zn (CuZn-SOD). In general, Mn-SOD is located in mitochondria, Fe-SOD in chloroplasts, and CuZn-SOD in chloroplasts and the cytosol (Bowler *et al.*, 1994). Manganese-containing SOD is also present in the peroxisomes (Sandalio *et al.*, 1987).

Three types of SOD are distinguished according to their sensitivity to cyanide and H_2O_2. Activity of CuZn-SOD is sensitive to both H_2O_2 and cyanide, while Fe-SOD is not sensitive to cyanide, but H_2O_2 inhibits its activity. Mn-SOD is not affected by H_2O_2 or cyanide (Scandalios, 1993; Bowler *et al.*, 1994). Measurement of these isoforms of SOD is useful for determining the micronutrient status of plants, for example in Zn (Cakmak & Marschner, 1987; Cakmak *et al.*, 1997), Fe (Iturbe-Ormaetxe *et al.*, 1995) and Mn (Garcia *et al.*, 1981; Yu *et al.*, 1998).

In higher plants, CuZn-SOD is the most abundant SOD, while Mn-SOD and Fe-SOD form a smaller proportion of total SOD activity (Jackson *et al.*, 1978; Alscher *et al.*, 1997). In leaves of different wheat cultivars, deficient supply of Zn decreased total SOD activity and, more distinctly, CuZn-SOD activity, whereas Mn-SOD activity was not affected by Zn deficiency (Cakmak *et al.*, 1997, 1998). Recently, similar results were found by Yu *et al.* (1999) in various wheat genotypes differing in sensitivity to Zn deficiency. They measured both activity and concentration of CuZn-SOD using capillary electrophoresis, and suggested that CuZn-SOD can be used as a tool in assessing the genotypic variation in sensitivity to Zn deficiency. Despite severe decreases in CuZn-SOD activity, there is no compensatory increase in Mn-SOD activity in Zn-deficient plants. Cakmak *et al.* (1997), studying wheat, and Yu & Rengel (1999), working on lupin, found that Zn deficiency markedly reduced CuZn-SOD activity, but did not affect Mn-SOD activity. However, under Mn deficiency decreases in Mn-SOD were accompanied by increases in CuZn-SOD activity (Del Rio *et al.*, 1978; Yu & Rengel, 1999). As in leaves, Zn deficiency lowered SOD activity in roots of cotton plants, and these decreases in SOD activity were paralleled by increases in the amount of $O_2^{\bullet-}$ measured by ESR in the cytosol of root cells (Cakmak & Marschner, 1988a,b). Decreases in CuZn-SOD activity caused by Zn deficiency have also been shown in bean (Cakmak & Marschner, 1993; Wenzel & Mehlhorn, 1995), *Lemna gibba* (Vaughan *et al.*, 1982) and tobacco (Yu *et al.*, 1998).

Irrespective of the Zn nutritional status of plants, CuZn-SOD activity can be decreased when plants are exposed to a strong photooxidative stress or conditions producing high amounts of H_2O_2. In plant and animal cells, H_2O_2 is effective in inactivating and degrading CuZn-SOD (Strack *et al.*, 1996; Casona *et al.*, 1997). Inhibition of CuZn-SOD activity by H_2O_2 was ascribed to reduction of enzyme-bound Cu^{2+} to Cu^{+} by H_2O_2; the reduced Cu reacts with H_2O_2, resulting in production of $OH^{\bullet}$. This hydroxyl radical was suggested by Hodgson & Fridovich (1975) to be responsible for inactivation of CuZn-SOD. Inactivation of SOD can also occur in plants with high sensitivity to low temperature (Michalski & Kaniuga, 1981). This inactivation was attributed to an inhibitory action of H_2O_2 which is accumulated at low temperatures.

2. *H_2O_2-scavenging enzymes*

Higher sensitivity of Zn-deficient plants to oxidative stress conditions cannot be attributed soley to reduced CuZn-SOD activity. Zinc is also indirectly required for high activity of the enzymes involved in H_2O_2 detoxification such as catalase, ascorbate peroxidase and glutathione reductase. In Zn-deficient plants activity of catalase was reduced, although Zn is not a required cofactor for catalase activity. This decrease in catalase activity with Zn deficiency was assumed to be related to inhibition of

catalase by $O_2^{\bullet-}$ (Cakmak & Marschner, 1988a). The catalase enzyme is very sensitive to $O_2^{\bullet-}$ and can be inactivated by increased levels of $O_2^{\bullet-}$ (Fridovich, 1986). Because Zn deficiency strongly reduces protein synthesis (Kitagishi & Obata, 1986; Cakmak *et al.*, 1989; Marschner, 1995), biosynthesis of H_2O_2-scavenging enzymes can be impaired as a result of Zn deficiency. Accordingly, in bean plants Zn deficiency reduced the activities of glutathione reductase and ascorbate peroxidase in young leaves, although these enzymes do not need Zn for their activity (Cakmak & Marschner, 1993). Consequently, because of the reduced activity of enzymes scavenging $O_2^{\bullet-}$ and H_2O_2, enhanced production of $OH^{\bullet}$ (via the Haber–Weiss reaction) can be expected in Zn-deficient plants. However, concentrations of $OH^{\bullet}$ in Zn-deficient plants have not been reported.

V. INVOLVEMENT OF ZINC IN PLANT STRESS TOLERANCE

As SODs play critical roles in the oxidative defence systems of all biological tissue (Bowler *et al.*, 1992, 1994; Scandalios, 1993), it can be suggested that plants with reduced SOD activity (i.e. marginal or severe Zn deficiency) should be highly sensitive to oxidative stress factors such as drought, chilling, O_3, pathogenic infection and salinity. There are results which support this suggestion. In bush bean, Zn deficiency caused a high sensitivity to O_3 toxicity (Wenzel & Mehlhorn, 1995). Ozone is known to exert its deleterious effect on plant cells by producing toxic O_2 species such as $O_2^{\bullet-}$ (Sharma & Davis, 1997). The higher O_3 sensitivity of Zn-deficient bush bean plants correlated well with a reduced activity of CuZn-SOD, prompting Wenzel & Mehlhorn (1995) to suggest that CuZn-SOD plays an important role in plant defence systems against O_3 toxicity. Similarly, overproduction of CuZn-SOD in the cytosol in response to O_3 exposure protected transgenic tobacco plants from O_3 damage (Pitcher & Zilinskas, 1996). Mn-SOD and Fe-SOD were also overexpressed in chloroplasts from plants exposed to O_3, thereby contributing to plant tolerance against O_3 toxicity (Van Camp *et al.*, 1994, 1996).

Besides protecting against O_3 toxicity, increased activity of CuZn-SOD also enhanced plant resistance to other oxidative stress factors. An NaCl-tolerant cultivar of pea, compared with a sensitive cultivar, had enhanced chloroplastic and cytosolic CuZn-SOD activity when treated with NaCl (Hernandez *et al.*, 1995). In transgenic tobacco plants, salt stress was associated with overexpression of cytosolic and chloroplastic CuZn-SOD enzymes (Van Camp *et al.*, 1996). Drought stress represents another oxidative environmental factor causing enhanced production of $O_2^{\bullet-}$ and H_2O_2, particularly in chloroplasts (Price & Hendry, 1989, 1991; Smirnoff, 1993; Bohnert *et al.*, 1995). In pea plants, decreases in leaf water potential caused by withholding water resulted in higher cytosolic and chloroplastic CuZn-SOD activity. This was considered an adaptive mechanism for minimizing drought-related cell damage in plants (Mittler & Zilinskas, 1994).

Increased activity of CuZn-SOD and its involvement in stress tolerance has also been shown in plants affected by low temperatures. Chilling stress, in combination with higher light intensity, is responsible for extensive photooxidative damage to chloroplasts by ROS (Wise & Naylor, 1987; Long *et al.*, 1994). Such photooxidative damage was greatly decreased in transgenic tobacco plants that were capable of overexpressing genes encoding the chloroplastic CuZn-SOD enzymes (Sen Gupta *et al.*, 1993a,b). Overexpression of CuZn-SOD genes was also demonstrated in plants exposed to herbicide treatment (Perl *et al.*, 1993), sulphur dioxide (Madamanchi *et al.*, 1994), UV-B radiation (Willekens *et al.*, 1994; Rao *et al.*, 1996) and pathogenic infection (Mittler *et al.*, 1996; Fodor *et al.*, 1997). As shown under field conditions, chilling stress in citrus and drought stress in wheat became more pronounced in plants suffering from Zn deficiency (Cakmak *et al.*, 1995; Ekiz *et al.*, 1998). These results may be due to reduced activity of enzymes scavenging $O_2^{\bullet-}$ and H_2O_2 in Zn-deficient tissues. In wheat plants, decreases in grain yield due to drought stress were more marked when plants were Zn-deficient (Ekiz *et al.*, 1998). By affecting the synthesis and activity of antioxidative enzymes, Zn is an important factor in plant defence systems against destructive O_2 species. Thus improvement of the Zn nutritional status of plants may be of great importance for their survival under oxidative stress conditions (drought, chilling, high light levels, ozone and salinity).

VI. CONCLUSIONS

This review presents several lines of evidence demonstrating that enhanced production of ROS is primarily involved in Zn-deficiency-induced impairments in cellular function and integrity. Zinc performs various important roles in protecting cells from the damaging reactions caused by ROS. As summarized in Fig. 4, Zn is directly or indirectly required for scavenging $O_2^{\bullet-}$ and H_2O_2, and thus for blocking generation of the powerful oxidant $OH^{\bullet}$. In Zn-deficient cells, Fe accumulation is substantial and results in a high physiological demand for Zn, particularly at membrane-binding sites for Fe. In the absence of Zn, binding of Fe to membrane constituents or DNA leads to site-specific production of $OH^{\bullet}$. Zinc also interferes with $O_2^{\bullet-}$-generating oxidases located in plasma membranes. Zinc is particularly needed within the environment of plasma membranes to maintain their structural and functional integrity. These Zn-deficiency-

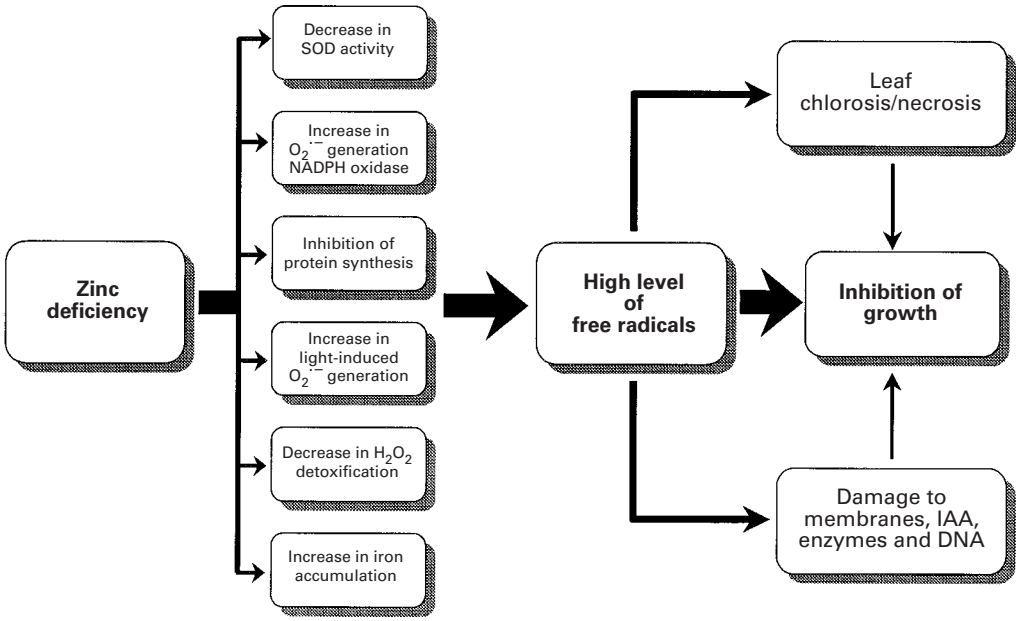


Fig. 4. Major physiological changes occurring in Zn-deficient cells and the effect on plant growth.

related disturbances in cellular metabolism are responsible for oxidative damage of membrane proteins, phospholipids, chlorophyll, nucleic acids, SH-containing enzymes and IAA, and thus inhibition of plant growth. Recent reports demonstrate that shoot and root meristematic activities of plants are rapidly blocked under oxidative stress conditions as a result of DNA damage (Reicheld *et al.*, 1999). Very high concentrations of Zn in meristematic plant cells (Kitagishi & Obata, 1986; Hossain *et al.*, 1997) demonstrate the crucial roles played by Zn in highly metabolically active differentiating cells.

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