MANGANESE TOXICITY AND TOLERANCE IN COWPEA

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THE ROLE OF THE LEAF APOPLAST

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KURZZUSAMMENFASSUNG

Cowpea (*Vigna unguiculata*) zeigt charakteristische Mn-Toxizitätssymptome in Form kleiner brauner Punkte auf älteren Blättern, gefolgt von Chlorosen, Nekrosen und Blattfall. Die verbräunten Epidermiszellwände weisen Akkumulationen von Mn^{IV} und oxidierten Phenolen auf. Die Oxidation wird vermutlich von Peroxidasen (PODs) unter Bildung der Intermediate Mn^{III} und Phenoxyradikalen katalysiert, die zu einer weiteren Schädigung des Gewebes führen können. Die durch POD katalysierte Reaktion ist vermutlich ursächlich für die Entstehung von Mn-Toxizität. Der Einfluss eines Mn-Überangebotes auf die Physiologie des Blattapoplast stand im Mittelpunkt der vorliegenden Arbeit.

(i) Ein Mn-Überangebot führt zu einer erhöhten Aktivität von zytoplasmatischen, zellwand-gebundenen und wasserlöslichen apoplastischen PODs. Die Abgabe von PODs in den Apoplasten ist ursächlich für die signifikante Erhöhung der POD-Aktivität in der AWF. Bei hohem Mn-Angebot konnte mit dem Auftreten erster brauner Punkte erhöhte POD-Aktivitäten nachgewiesen werden. Bei einer moderaten Mn-Gabe konnte erst nach dem Auftreten erster brauner Punkte eine signifikante Erhöhung der POD-Aktivität nachgewiesen werden. Zellwand-gebundenen PODs könnten somit ebenfalls an der Verbräunung der Zellwand beteiligt sein.

(ii) PODs katalysieren die Bildung sowie den Abbau von H_2O_2 im Apoplasten. Für die Bildung von H_2O_2 wird die Oxidation von NADH signifikant durch Mn^{II} und p-Coumarsäure stimuliert. Die geringfügige Erhöhung des Mn-Angebotes führte bereits vor dem Auftreten brauner Punkte zu einer Calloseinduktion und einer Erhöhung der spezifischen NADH-POD Aktivität. Die NADH-POD wird signifikant durch bislang unbekannte Substanzen der AWF beeinflusst. Phenolische Verbindungen des Apoplasten, die sich stimulierend im sensitiven und hemmend im toleranten Genotyp auswirken könnten, werden als Regulatoren für NADH-POD in Betracht gezogen.

(iii) Mn verursacht im Apoplasten oxidativen Streß. Dies wird duch die signifikanten Veränderungen der apoplastischen Ascorbinsäure (AA)-Konzentration angezeigt. Genotypen mit hohen AA-Konzentrationen im Apoplasten könnten sich als Mn-tolerant erweisen. Die Applikation von Ascorbinsäure über den Blattstiel führte zu einer Erhöhung der Mn-Gewebetoleranz. Einen einfachen Zusammenhang zwischen hohen Ascorbinsäurekonzentrationen im Apoplasten und Mn-Toleranz wird jedoch nicht in Betracht gezogen.

(iv) Durch die detaillierte Untersuchung des Apoplasten Proteoms mit Hilfe von 2D-Elektrophorese und LC-MS/MS-Analysen konnten zahlreiche Mn-induzierte Proteine charakterisiert werden. Die PR-ähnlichen Proteine werden bei fortgeschrittener Schädigung in den Apoplasten abgegeben und durch zahlreiche weitere abiotische Faktoren induziert. Die Induktion dieser Proteine durch Mn wird als generelle Streßanwort angesehen.

Aufgrund der vorliegenden Untersuchungen kann auf folgende Mn-induzierte Reaktionsabfolge im Apoplasten geschlossen werden:

Erhöhung der Mn-AWF-Konzentration→Callosebildung←→Stimulierung der H_2O_2-bildenden NADH-PODs→Aktivierung von zellwand-gebundenen und freien H_2O_2-abbauenden PODs→Oxidation von Mn und Phenolen; Bildung brauner Punkte→Abgabe von PR-ähnlichen Proteinen in den Apoplasten. Die Ursachen genotypischer Toleranzunterschiede sind noch ungeklärt. Jedoch könnte der Regulation von H_2O_2-bildenden PODs im Apoplasten durch Phenole eine entscheidende Rolle zukommen.

Schlagworte: Mangan-Toxizität, Blattapoplast, Cowpea

ABSTRACT

In cowpea (*Vigna unguiculata*), excess manganese (Mn) causes the formation of brown spots on older leaves, followed by chlorosis, necrosis and leaf shedding. The brown spots represent local accumulations of oxidized Mn (Mn^{IV}) and oxidized phenols in the cell wall, especially in the epidermis. Apoplastic peroxidases (PODs) were proposed to catalyse the oxidation of Mn^{II} and phenolic compounds, mediated by the formation of reactive Mn^{III} and phenoxyradicals ($PhO^{\cdot}$). The leaf apoplast was considered the most important compartment for development and avoidance of Mn toxicity and Mn tolerance. Therefore, investigations were focussed on the physiology of the apoplast and its modifications by excess Mn.

(i) Mn excess enhanced activities of cytoplasmic, cell wall-bound and total free H_2O_2-consuming guaiacol-PODs in the leaf apoplast. The increase of AWF-POD activity was mainly caused by an enhanced release of PODs into the leaf apoplast. At highly toxic Mn supply, POD activities in the apoplastic washing fluid (AWF) were increased concomitantly with the appearance of brown spots. But at moderately toxic Mn supply, a significant activation of PODs in the AWF was only observed at higher densities of brown spots. Therefore, it appears that cell wall-bound guaiacol-PODs are involved in the formation of brown depositions.

(ii) Peroxidases in the leaf AWF showed also H_2O_2-producing capacity. The production of H_2O_2 by the oxidation of the substrate NADH was significantly stimulated by Mn^{II} and p-coumaric acid. The specific activity of NADH-*per*oxidase and the callose formation were induced at very early stages of Mn toxicity prior to the formation of brown depositions. NADH-*per*oxidase activity in the AWF was modulated by non-protein compounds, most probably phenols, which stimulated or inhibited NADH-*per*oxidase activity in Mn-sensitive or Mn-tolerant leaf tissue, respectively.

(iii) Mn toxicity induced oxidative stress in the leaf apoplast as indicated by changes in the ascorbic acid (AA) homeostasis in the AWF. Genotypic higher Mn tolerance was related to the capacity to maintain higher AA levels in the AWF, and application of AA solutions via the petiole enhanced Mn tolerance. However, overall the results suggest a contributing but not a decisive role of apoplastic AA in the control of Mn toxicity.

(iv) A detailed study of the apoplast proteome by two-dimensional gelelectrophoresis followed by massspectrometric identification of the proteins revealed a Mn-induced release not only of PODs but also of pathogenesis related-like (PR-like) proteins. Since the release of proteins is a rather late response to toxic Mn, and several biotic and abiotic factors induce the expression of similar PR-like proteins, this response is considered a general stress response.

Based on the results the following sequence of events induced by toxic Mn supply is proposed:

increase in apoplastic free Mn^{2+} → callose formation ←→ stimulation of apoplastic H_2O_2-producing NADH-*per*oxidase → activation of cell wall-bound and soluble H_2O_2-consuming PODs → oxidation of Mn^{II} and phenolics leading to the formation of brown depositions → release of PR-like proteins into the apoplast.

For the not yet well understood genotypically enhanced Mn tolerance, the control of H_2O_2-producing PODs in the leaf apoplast by phenolic compounds seems to play an important role.

Keywords: Manganese toxicity, leaf apoplast, cowpea

CONTENTS

ABBREVIATIONS

1D	one-dimensional
2D	two-dimensional
AA	ascorbic acid
AAO	ascorbic acid oxidase
ACN	acetonitrile
AWF	apoplastic washing fluid
BN	blue native
CAT	catalase
cv.	cultivar
DHA	dehydroascorbic acid
DHAR	dehydroascorbate reductase
ESI	electrospray ionisation
GR	glutathione reductase
HPLC	high performance liquid chromatography
HR	hypersensitive response
ICP-OES	inductive-coupled plasma optical emission spectroscopy
IEF	isoelectric focussing
IPG	immobilized pH gradient
LC-MS	liquid chromatography - mass spectrometry
MDHA	monodehydroascorbate reductase
MDHAR	monodehydroascorbic acid reductase
MWCO	molecular weight cut off
n.s.	not significant
PCD	programmed cell death
POD	peroxidase
PPO	polyphenoloxidase
PR	pathogenesis related
SAR	systemic acquiered resistance
TOF	time of flight
TVu	tropical vigna unguiculata
v	volume
w	weight

GENERAL INTRODUCTION

The heavy metal manganese (Mn, density 7.43 g cm^{-3}) is an essential trace element and involved in various physiological processes in the plant tissue (Campbell and Nable, 1988). The perhaps most important role of Mn in nature is its involvement in oxygen evolution in plants (Amesz, 1983; Prince, 1986). Manganese is an integral part of several enzymes, e.g. PSII, Mn-SOD, purple acid phosphatase (Burnell, 1988), germin-like protein (Requena and Bornemann, 1999) and acts as co-factor for more than 30 enzymes (Burnell, 1988). The amount of Mn in soils varies from 20 to 3000 mg kg^{-1} and its availability for plants is dependent from the level of easily reducible Mn oxides, the pH value in soils, the redox potential, the availability of further nutrients and the presence of Mn-oxidizing and Mn-reducing microorganisms (Ghiorse, 1988; Reisenauer, 1988). For optimal plant growth and development, plants have to accumulate at least 30 mg Mn per kg dry weight in tissues, regardless of plant species and cultivars (Bergmann und Neubert, 1976; Reisenauer, 1988; Marschner, 1995).

Under condition of increased Mn availability, e.g. in acid, insufficiently drained soils with low redox potential (Foy, 1984; Sparrow and Uren, 1987; Fox et al., 1991), under conditions such as drought, heat and after steam sterilization of glass-house soils (Siman et al., 1974; Sonneveldt and Voogt, 1975; Grasmanis and Leeper, 1988), high Mn concentrations in the plant tissue induces the formation of Mn toxicity symptoms and the reduction of plant growth. Worldwide, excess Mn causes losses of crop yield, predominantly on acid soils in the tropics and subtropics. Control measures such as soil amelioration by liming and soil drainage are often not economic, ecologically sound, and only partly successful (Schlichting and Sparrow, 1988). Since about 1980, the selection and breeding of Mn-resistant cultivars has been considered a potent strategy to face the problems of agriculture in developing countries. Plant species (Andrew and Hegarty, 1969; Horiguchi, 1987; Hannam and Ohki, 1988; Singer and Havill, 1993) and cultivars within species (Nelson, 1983; Moroni et al., 1991; Sale et al., 1992; Mgema and Clark, 1995) show a high variability in Mn resistance. A particular high genetic variability was observed in the Mn-sensitive legumes soybean (*Glycine max.*) (Carter et al., 1975; Heenan and Carter, 1975), common bean (*Phaseolus vulgaris*) (Horst and Marschner, 1978) and cowpea (*Vigna unguiculata*) (Horst, 1980).

The plant strategy to prevent Mn toxicity is yet only scarcely understood. The restriction of root-to-shoot transport of heavy metals was considered an avoidance mechanism (Verkleij and Schat, 1990). The inhibition of Mn uptake or retention of Mn in the root is not a common plant strategy to maintain normal growth and to suppress the expression of toxicity symtoms in spite of high Mn supply (Andrew and Hegarty, 1969; Horst, 1980). For instance, a high accumulation of Mn in roots combined with low Mn tissue concentrations in the shoots was found in Mn-sensitive tobacco (Wang et al., 1992). However, in most cases considerable differences in the expression of Mn toxicity occurred frequently at similar Mn tissue contents in leaves (Horst, 1980). Mn-tolerant plant tissues sometimes even showed higher Mn contents than susceptible tissues (Macfie and Taylor, 1992; Wang et al., 1992). It, therefore, appears that the ability to avoid Mn toxicity is predominantly based on a higher leaf tissue Mn tolerance. The sequestration and translocation of Mn into vacuoles (Hirschi et al., 2000; Schaaf et al., 2002) and ER (Wu et al., 2002) was proposed to enhance Mn tolerance. In cowpea, the hypothetical sequestration of Mn by organic acid and the translocation in the vacuole could not satisfactorily explain genotypically differences in Mn tolerance (Maier, 1997; Horst and Maier, 1999; Horst et al., 1999). The association of heavy metals to phytochelatins with following translocation into the vacuole is known as a potent detoxification mechanism (Steffens, 1990), but a Mn-induced phytochelatin formation has not yet not be observed (Rauser, 1995). In addition to genetically determined differences in Mn tolerance, several developmental, environmental and nutritional factors affect the Mn sensitivity of plant tissues (Horst, 1988). Within plants, older leaves are more susceptible to Mn than younger leaves (Horst, 1982). The application of Si (Williams and Vlamis, 1957; Horst and Marschner, 1978; Iwasaki et al., 2002a,b; Rogalla and Römheld, 2002) and NO_3-N compared to NH_4-N nutrition enhanced Mn tolerance (Maier, 1997). The light intensity has also an significant effect on Mn tissue tolerance, but conflicting results were reported: shaded leaves of cowpea (Wissemeier and Horst, 1992) were more Mn-sensitive than light-exposed leaves, whereas leaves of common bean exposed to high-light intensities showed a stronger expression of Mn toxicity than shaded leaves (González et al., 1998). In all cases higher Mn tolerance due to specific environmental and nutritional conditions were not due to lower Mn concentrations in the leaf tissue.

Manganese belongs to the group of borderline ions (Nieboer and Richardson, 1980), which were generally involved in a direct or indirect generation of free radicals or reactive oxygen species (Dietz et al., 1999). Transition metals, particular iron and copper were shown to promote the hydroperoxide heterolytic O-O bond cleavage causing the formation of highly aggressive $^{\cdot}OH$ (Fenton reaction), but the formation of $^{\cdot}OH$ by Mn^{2+} could not be demonstrated (Halliwell and Gutteridge, 1984; Cheton and Archibald, 1988).

During last 40 years of research on the physiology of Mn toxicity, several physiological systems in the plant tissue were considered to be affected by excess Mn (see reviews Horst et al., 1988; El-Jaoual and Cox, 1998) and several investigations were focussed on Mn excess-induced processes in the symplast. An impact of excess Mn on photosynthetic rate and chlorophyll concentration (Ohki, 1985; Nable et al., 1988; Moroni et al., 1991; Macfie and Taylor, 1992; González et al., 1998), ribulose-bisphosphate carboxylase/oxygenase activity (Rubisco) (Houtz et al., 1988; McDaniel and Toman, 1994) and CO_2 assimilation (González and Lynch, 1997) was documented. Hypothesis for explaining the inhibition of net photosynthesis by excess leaf Mn^{2+} include: (i) chloroplastic enzymes are inhibited by non specifically increased concentrations of polyphenolic oxidation products arising via an observed increase of polyphenoloxidase activity, (ii) the indirect or direct disruption of chloroplast membranes, and (iii) the replacement of Mg^{2+} and formation of rubisco-Mn^{2+} complexes (Houtz et al., 1988 and cited within). Excess Mn affected the indoleacetic acid oxidase system (IAA-oxidase) (Morgan et al., 1966; Taylor et al., 1968) and the activity of enzymes, e.g. peroxidase, catalase (Sirkar and Amin, 1974; Leidi et al., 1987; Nable et al., 1988), superoxide dismutase (Leidi et al., 1987; González et al., 1998) and polyphenol oxidase (Sirkar and Amin, 1974; Nable et al., 1988). The pool of antioxidants (ascorbic acid, dehydroascorbate and glutathione) and related enzymes (ascorbic acid oxidase, cytochrom c oxidase, glutathion oxidase, ascorbate peroxidase, glutathione peroxidase, glutathion reductase) was also strongly affected by excess Mn, indicating the occurrence of oxidative stress (Gonzáles et al., 1998). The replacement of metal cofactors by Mn^{2+} as the cause for inactivation of antioxidative enzymes was discussed (Leidi et al., 1987; Nable et al., 1988). The binding of heavy metals to O-, N- and S-containing ligands is also considered suppressing antioxidative compounds and enzymes (Dietz et al., 1999; Schützendübel and Polle, 2002), but this is probably not the overall cause of Mn-induced oxidative stress (Gonzáles et al., 1998). In conclusion, on

the basis of the available information a general picture on the physiology of Mn toxicity is difficult to draw. Moreover, different cultivars and leaves differing in age were used for the investigations. The nature of Mn toxicity symptoms varies by cultivar and leaf age (see review Horst, 1988; El-Jaoual and Cox, 1998) and, therefore, the results are difficult to compare.

However, many plant species show first visible Mn toxicity symptoms by the expression of small dark brown spots on stems and older leaves (Horst, 1988; Fig. 1A). Several stages of Mn toxicity symptoms occur with prolonged Mn treatment. The formation of brown spots is followed by the occurrence of chlorosis (Fig. 1B), necrotic lesions (Fig. 1C), and leaf shedding.

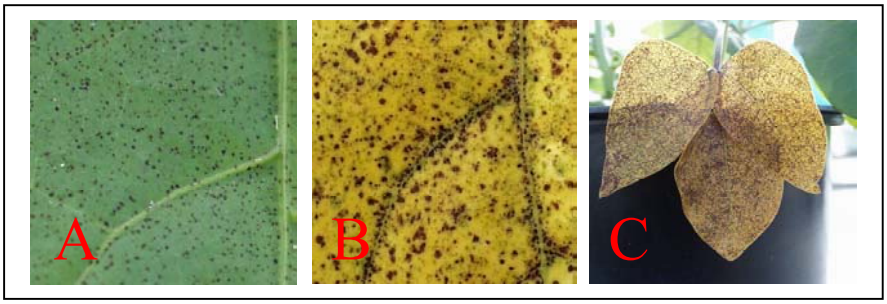

Figure 1 Different stages of Mn toxicity in cowpea. At excess Mn, first visible symptoms are brown spots on older leaves (A), followed by chlorosis (B) and necrosis (C)

The microscopic view of visible brown spots on older leaves reveals that these spots are formed by brown depositions in cell walls (Fig. 2).

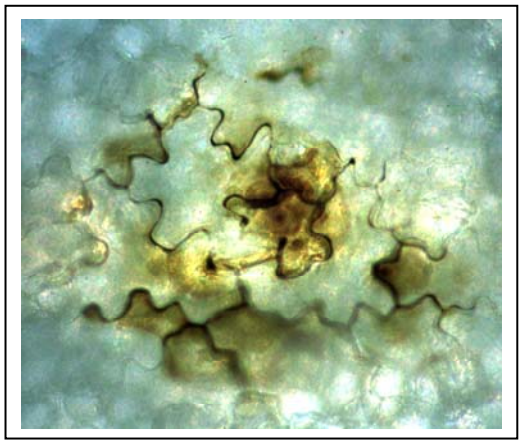

Figure 2 Microscopic view (200x) of a macroscopic visible brown spot in a leaf of a Mn treated cowpea plant (*Vigna unguiculata*).

In several plants, brown depositions were preferential formed on the base of trichomes (Bussler, 1958; Blamey et al., 1986; Horiguchi, 1987). In *Phaseolus vulgaris*, particular epidermal cells close to leaf veins showed firstly such a local browning induced by excess Mn (Horst and Marschner, 1978).

Since these visible Mn toxicity symptoms express in the leaf apoplast, this compartment was considered to be most important for development and avoidance of Mn toxicity (Horst, 1988). The plant apoplast is the extraprotoplastic matrix including cell walls (Dietz, 1997) and was once described as "the dead excursion product of the living protoplast" (see review Sattelmacher, 2001). At the beginning of the last century, the apoplast was considered a "dead" water transportation continuum (see review Sakurai, 1998). Today it is known that the apoplast is a complex space for nutrient uptake, short- and long-distance transport, a compartment for storage and reactions and a habitat for microorganisms (Sattelmacher, 2001) and various ions, metabolites, plant growth regulators and proteins are located in the apoplast (Dietz, 1997).

The presence of Mn oxides in the cell wall, especially in the area of brown deposits, was demonstrated by histochemical investigations using Arnold´s reagent (tetramethyl-diaminediphenylmethane; Bussler, 1958; Andrees, 1971; Horst and Marschner, 1978) and benzidine (Horiguchi, 1987; Wissemeier and Horst, 1992). Also autoradiography studies using ^{54}Mn showed an uneven distribution of Mn in Mn-sensitive cultivars of *Phaseolus vulgaris* and *Vigna unguiculata*. Furthermore, the local accumulations of ^{54}Mn in the leaf tissue were congruent with brown depositions (Horst and Marschner, 1978; Horst, 1980). However, the treatment of these spots with hydroxylamine hydrochloride, which readily reduces and dissolves $Mn^{IV}O_2$, decolourised the brown spot only slightly. A decolourisation was only observed by addition of thioglycolic acid, indicating the presence of oxidized phenolic compounds (Wissemeier and Horst, 1992).

The uneven distribution and accumulation of Mn^{IV} in the leaf tissue was interpreted as Mn tolerance mechanism (Bussler, 1958; Blamey et al., 1986; Le Mare, 1977; Horiguchi, 1987). But the absence of brown depositions in Mn-tolerant leaf tissues indicates that the enhanced Mn oxidation is a characteristic event in Mn-sensitive tissues (Horst, 1988). The density of these brown spots is a reliable parameter for the quantification of the severity of Mn toxicity (Wissemeier and Horst, 1991).

In general, the oxidation of phenolic compounds with consequent formation of brown depositions in plant tissues occurres in response to wounding (Tomas et al., 1997; Campos-Vargas and Saltveit, 2002; Cantos et al., 2002), pathogen infection (Chen and

Seguin, 1999; Kalim et al., 2003) and nutrient deficiency (Cakmak and Römheld, 1997). Peroxidases (POD) and polyphenol oxidases (PPO) are involved in stress-induced browning of tissue (Lagrimini and Rothstein, 1987; Lagrimini, 1991; Cakmak and Römheld, 1997). Particular POD was considered to be involved in Mn-induced browning of leaf cell walls (Sirkar and Amin, 1974; Horiguchi, 1987; Horiguchi, 1988; Wissemeier, 1988). The pioneering work of Kenten and Mann (1950) showed a close relationship between the oxidation of Mn^{II} and phenolic compounds by horseradish peroxidases, accompanied by the formation of reactive phenoxy radicals ($PhO^{\cdot}$) and Mn^{III} leading to metabolic distortions and leaf necrosis (Kenten and Mann, 1956). This concept of Mn-induced injury of plant tissues has been basically adopted in more recent work on Mn toxicity (Horiguchi, 1987; Horst, 1988; Wissemeier and Horst, 1992, Horst et al., 1999).

Plant peroxidases (EC 1.11.1.7) are monomeric heme-containing enzymes with differences in molecular mass, isoelectric point, pH optima, substrate specificity, function, and are widely distributed in plant tissues. They are involved in a number of physiological reactions, e.g. lignification, suberization, auxin catabolism, wound healing caused by biotic and abiotic stresses, and defence against pathogen infection (Campa, 1991; Hiraga et al., 2001). A multitude of stresses affects the level of POD activity in the plant tissue and therefore, plant PODs were frequently used as a biochemical marker (Castillo, 1986). Especially apoplastic PODs respond sensitive to stress induced by ozone (Castillo and Greppin, 1986; Peters et al., 1988; Ranieri et al., 2003), pathogen attack (Bestwick et al., 1998), increased Zn supply (Brune et al., 1994) and NaCl (Lin and Kao, 2001). In the plant apoplast, PODs were attributed to act as H_2O_2-consuming and phenol-oxidizing enzymes, thus leading to secondary cell-wall and lignin formation (Polle et al., 1994; Sato et al., 1995; Christensen et al., 1998; Kärkönen et al., 2002). A stimulating effect of excess Mn on cytoplasmic and cell wall-bound PODs was documented (Horst, 1988; Horst et al., 1999). Based on the publications of Kenten and Mann (1950), the proposed key reactions of Mn toxicity in susceptible tissues are displayed by a simplified scheme (Fig. 3).

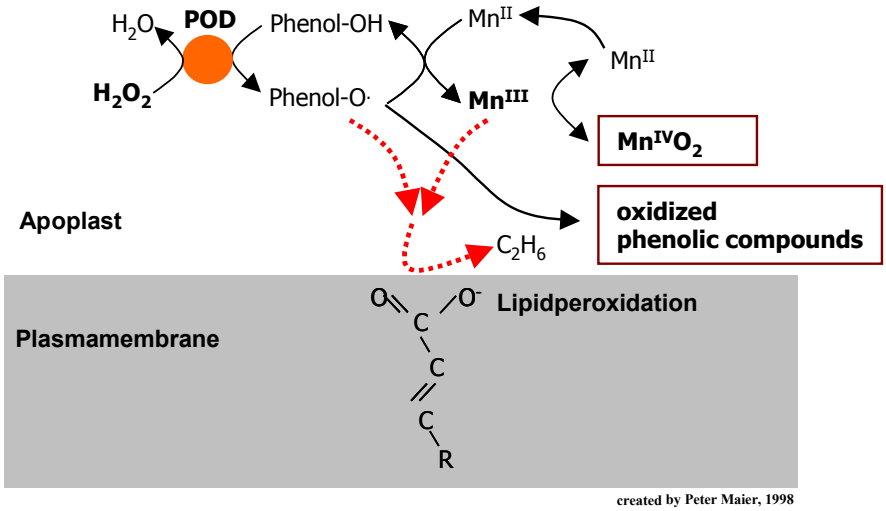

Figure 3 Simplified scheme of the proposed Mn-induced reactions in the leaf apoplast. The oxidation of phenolic compounds by peroxidase (POD) is enhanced particularly in Mn-sensitive leaf tissue and accompanied by the oxidation of Mn^{II}. Oxidation of Mn^{II} and phenolic compounds causes the formation of phenoxy radicals and Mn^{III}. These reactive species might induce further redox reactions in the leaf apoplast, e.g. lipid peroxidation, causing ethylene formation (Horst, 1988). The primary oxidation product Mn^{III} might disproportionate to Mn^{II} and Mn^{IV}. Oxidized Mn^{IV} accumulates in the cell wall, together with oxidized phenolic compounds leading to the formation of brown depositions in the cell wall.

During POD-catalysed oxidation of phenolic compounds, phenoxyradicals ($PhO^{\cdot}$) and Mn^{III} are presumably formed and are proposed as primary toxic compounds in the leaf apoplast at excess Mn (Horst et al., 1999). The POD-catalysed formation of brown depositions was considered the key reaction in Mn toxicity. Beside the catalysis of cross-linking processes in the cell wall, PODs are also involved in the H_2O_2 formation in the leaf apoplast, needed for lignification or contributing to the "oxidative burst" (Elstner and Heupel, 1976; Gross et al., 1977; Mäder et al., 1980; Mäder and Amberg-Fisher, 1982; Halliwell, 1978; Bolwell et al., 1995). The activity of H_2O_2-producing PODs is strongly dependent on the co-factors Mn and phenolic compounds (Elstner and Heupel, 1976; Gross et al., 1977). Phenolic compounds may stimulate or inhibit H_2O_2-producing POD depending on their nature (Pedreño et al., 1987). In the leaf apoplast of cowpea, excess Mn might stimulate H_2O_2 formation. This is indicated by the increased H_2O_2 formation by intact washed leaf segments from Mn-treated plants of Mn-sensitive leaf tissue (Horst et al., 1999). Due to the close relationship between the activity of H_2O_2-producing PODs and the co-factor Mn, PODs represent a potential source for Mn-stimulated H_2O_2 formation in the apoplast.

The POD-catalysed reactions are partially controlled by ascorbic acid (AA). AA affects the functionality of H_2O_2-producing (Otter and Polle, 1994) and H_2O_2-consuming peroxidases; the oxidation of phenolic compounds by PODs in the apoplast is

suppressed by AA (Takahama and Oniki, 1992; Takahama, 1993; Sánchez et al., 1997). For the POD-catalysed oxidation of phenolic compounds, AA acts as the secondary electron donator by reducing phenoxyradicals, resulting in an inhibition of the radical chain reaction of phenol oxidation. In general, AA acid is a common antioxidant in plants (Schmitz and Noga, 2000) and its important role in stress resistance, growth, and regulation of cell signalling has been widely documented (Polle and Rennenberg, 1993; Noctor and Foyer, 1998; Horemans et al., 2000; Smirnoff, 2000; Pignocchi and Foyer, 2003). Plants with high AA-concentrations in the plant tissue and particularly in the leaf apoplast were considered less sensitive to oxidative damage by ozone than plants with low AA concentrations (Lee et al., 1984; Lee, 1991; Burkey and Eason, 2002). An effect of Mn on the antioxidant pool in the leaf tissue of cotton (Sirkar and Amin, 1974) and common bean (Gonzáles et al., 1998) has been demonstrated earlier. Therefore, high AA levels in the leaf tissue and particular the control of a high reduction capacity by maintaining high AA levels in the leaf apoplast might contribute to Mn leaf-tissue tolerance (Horst et al., 1999). This would require a sufficient AA production, a high regeneration rate in the symplast and an efficient transport of AA across the plasma membrane into the apoplast.

In general, antioxidants can be classified in (i) free radical terminators, (ii) chelators of metal ions and (iii) oxygen scavengers. Phenolic antioxidants are included into the category free radical terminators (Shahidi and Wanasundara, 1992), but were also proposed to elevate metal tolerance by sequestration of metal ions (Heim et al., 2001; Kidd et al., 2001; Lavid et al., 2001). Aoba (1986) proposed a positive correlation between Mn tolerance and the quantity of phenols in the plant tissue. He hypothesised that the presence of polyphenols determines the Mn tolerance of plant tissues. This was indicated by the strong increase of Mn tolerance of epidermal cells of onion scale in the presence of epigallocatechin or catechin/gallic acid, phenols abundantly present in Mn-tolerant tea leaves. Phenolic compounds have been shown to greatly affect POD-catalysed oxidation of Mn^{II} (Kenten and Mann, 1950): monohydroxy phenols but not dihydroxy and trihydroxy phenols stimulated the oxidation of Mn. Manganese affects the pool of phenolic compounds in the leaf tissue, because Mn significantly influences the Shikimate pathway and subsequent steps of the biosynthesis of plant secondary metabolites (see review Burnell, 1988). Based on these observations, interactions between phenolic compounds and excess Mn presumably influence Mn toxicity and Mn tolerance at different levels. More detailed studies on phenol contents and phenol

composition of leaf tissues differing in Mn tolerance are necessary to elucidate the role of phenolic compounds in Mn toxicity and Mn tolerance.

During last 20 years, studies on the physiology of Mn toxicity and Mn tolerance by Horst and his co-workers focussed on cowpea (*Vigna unguiculata* L.). With over 25% protein contents in seeds and leaves, cowpea is an important source of protein, minerals and vitamins in the daily diet of people, especially of rural and urban poor regions in Africa and South America. Cowpea is grown in over 60 countries worldwide and Nigeria is the largest producer and consumer of cowpea with about 5 million ha and over 2 million tons production (Singh et al., 2003).

The most recent investigations on the physiology of Mn toxicity and Mn tolerance of cowpea (*Vigna unguiculata* L.) are presented in this work. The leaf apoplast is considered the most important compartment for development and avoidance of Mn toxicity. Therefore, investigations were mainly focussed on this leaf compartment.

The presented study focuses on five areas, which are considered to particularly contribute to a better understanding of the role of the apoplast in Mn toxicity and Mn tolerance:

(i) The effect of excess Mn on peroxidases in several fractions of the leaf tissue (chapter 1).

(ii) The effect of excess Mn on the apoplast proteome (chapter 2).

(iii) The effect of Mn, phenolic compounds and apoplastic water-soluble non-protein compounds in the leaf apoplast on the functionality of PODs and their role in Mn toxicity and Mn tolerance (chapter 3).

(iv) The role of ascorbic acid in Mn tolerance (chaper 1 and 4).

(v) The sequence of events in the development of Mn toxicity (chapter 2 and 5).

CHAPTER 1:

APOPLASTIC PEROXIDASES AND ASCORBATE ARE INVOLVED IN MANGANESE TOXICITY AND TOLERANCE OF *VIGNA UNGUICULATA* L. WALP.

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ABSTRACT

Excessive manganese (Mn) supply induced the formation of brown spots on leaves as typical Mn toxicity symptoms in cowpea (*Vigna unguiculata* L. Walp.) grown in hydroponics. Differences in Mn resistance between cv. TVu 91 (Mn-sensitive) and cv. TVu 1987 (Mn-tolerant) expressed in the density of brown spots in older leaves were due to higher Mn tissue tolerance. Apoplastic water-soluble peroxidase (POD) in the apoplastic washing fluid (AWF) was enhanced by increasing Mn leaf contents and generally significantly higher in leaves of cv. TVu 91 than in cv. TVu 1987. Electrophoresis of AWF revealed the presence of several water-soluble POD isoenzymes. At toxic Mn supply, the activities of these and additional POD isoenzymes increased more in the Mn-sensitive cultivar.

Levels of ascorbic acid in the apoplast and cytoplasm of the Mn-sensitive cv. TVu 91 decreased with increasing leaf Mn contents, whereas Mn-tolerant cv. TVu 1987 was not affected. Mn treatment led to a stimulation of the enzymes of the ascorbic acid regeneration system (monodehydroascorbic acid reductase and glutathione reductase) in both cultivars. But the activation of glutathione reductase was clearly more enhanced in the Mn-tolerant cultivar TVu 1987.

The results provide circumstantial evidence that apoplastic ascorbate and peroxidases are involved in the expression of Mn toxicity and genotypic Mn tolerance.

INTRODUCTION

Manganese (Mn) toxicity is a major factor limiting plant growth especially on acid soils of the tropics and subtropics. Plant species and cultivars differ considerably in resistance to excess Mn. Cultivar specific differences in Mn resistance of cowpea are due to higher Mn tissue tolerance (Horst, 1980). First visible symptoms are brown spots on leaves followed by chlorosis, necrosis, and shedding of leaves. The brown spots include oxidized Mn (Horst and Marschner, 1978) and oxidized phenols (Wissemeier und Horst, 1992) in the cell wall of the epidermis. Oxidation of Mn^{II} in the apoplast has been proposed as the key reaction leading to Mn toxicity (Horst, 1988), because Mn^{III} may react as a powerful oxidant of proteins and lipids (Archibald and Fridovich, 1982a). Kenten and Mann (1950)

found a close relationship between the oxidation of Mn in the presence of peroxidase (POD) and phenols. The POD-catalyzed formation of polyphenols, which are responsible for the browning of the leaves, is accompanied by the formation of phenoxyradicals (Takahama and Oniki, 1992; Takahama, 1993).

Ascorbic acid is an important antioxidant in plants as are tocopherol, carotinoides and phenols (Polle and Rennenberg, 1993; Horemans et al., 2000; Schmitz and Noga, 2000; Smirnoff, 2000). It is an effective scavenger for phenoxy radicals by reducing these radicals and thus inhibiting phenol oxidation (Takahama and Oniki, 1992; Takahama, 1993; Sanchez et al, 1997). The involvement of an antioxidant system including an ascorbic acid regeneration-system (Castillo and Greppin, 1988; Smirnoff, 2000) in protecting plants against oxidative stress induced by ozone (Mehlhorn et al., 1987; Castillo and Greppin, 1988), heavy metals (Chaoui et al., 1997; Gupta et al., 1999) and pathogen infection, especially in the apoplast was described by Vanacker et al. (1998a,b). Gonzáles et al. (1998) suggested that Mn toxicity in common bean may be mediated by oxidative stress and that genotypic Mn tolerance may be related to the maintenance of higher ascorbate levels in the leaf tissue under Mn excess.

Provided that Mn toxicity is linked to the formation of phenoxy radicals and highly toxic Mn^{III} (Horst et al., 1999) we assume that the leaf antioxidant capacity could be responsible for the described differences in Mn tolerance of cowpea. Based on this hypothesis we tested the effect of Mn on the ascorbic acid status in the intercellular space of leaves and in the bulk-leaf tissue. Additionally, we monitored the activities of the ascorbic acid regenerating enzymes monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR). To define the role of peroxidase in Mn toxicity, the effect of Mn on the activities of cytoplasmic and apoplastic water-soluble, as well as ionically and covalently cell-wall bound POD extracted from two cowpea cultivars differing in Mn tolerance was studied.

MATERIALS AND METHODS

Cowpea (*Vigna unguiculata* (L.) Walp.) cultivars TVu 91 and TVu 1987 were grown hydroponically in a growth chamber under controlled environmental conditions at 30/25°C day/night temperature, 75±5 % relative humidity and a photon flux density of 270 µmol $m^{-1}s^{-1}$ photosynthetic active radiation at mid-plant height during a 16 h photoperiod. After germination in 1 mM $CaSO_4$, one seedling per cultivar was transferred to a constantly aerated nutrient solution in a 5L plastic pot. The composition of the basic nutrient solution

was [µM]: $Ca(NO_3)_2$ 1000, KH_2PO_4 100, $MgSO_4$ 325, FeEDDHA 20, NaCl 10, H_3PO_3 8, $MnSO_4$ 0.2, $CuSO_4$ 0.2, $ZnSO_4$ 0.2, Na_2MoO_4 0.05. After 15 days of preculture, the $MnSO_4$ concentration in the nutrient solution was increased to 50 or 100 µM Mn for 6 days, whereas control plants received 0.2 µM Mn continuously. Five replicates were used. Nutrient solutions were changed two to three times a week to maintain the nutrient supply.

For the quantification of Mn toxicity symptoms, the number of brown spots was counted on a 1 cm^2 area at the base, middle and tip on the upper side of the trifoliate leaf prior to harvest.

Apoplastic washing fluid (AWF) was extracted by a vacuum infiltration/centrifugation technique. Leaves were infiltrated with water by reducing the pressure to 35 hPa followed by a slow relaxation for 2 min. AWF was recovered by centrifugation at 1324*g* for 5 min at room temperature. AA and DHA concentrations in the AWF were measured immediately. For further analysis the AWF was stored at –20°C.

For measurements of AA and DHA in the leaf tissue, leaves were homogenized in 2% metaphosphoric acid, centrifuged at 5,000*g* for 15 min at 2°C. The supernatant was used for analysis immediately after centrifugation.

For the detection of the activities of MDHAR and GR from the leaf tissue, leaves were homogenized in 100 mM K_2HPO_4 pH 7.8 containing 0.5% (w/v) polyvinylpyrolidone (PVP), 5 mM ascorbic acid, and centrifuged (4,000*g*, 4°C) for 30 min. Supernatants were dialysed against 80% $(NH_4)_2SO_4$ for 1 h at 2°C. After dialyses, the extracts were centrifuged at 20,000g at 4°C for 10 min. The pellets were resuspended in a buffer containing 50 mM MES/KOH (pH 6.0).

For the extraction of POD-isoenzymes, approximately 0.5 g leaf material was homogenised in 3 ml 50 mM TRIS-MES (pH 7) with mortal and pestle on ice. After centrifugation at 5,000*g* at 4°C for 30 min, supernatants were used to determine cytoplasmic POD activity. Residues were washed with water and filtrated through a 0.11 µm nylon membrane (Millipore). The cell walls were transferred into a bag made of 40 µm nylon mesh and washed with 50 mM TRIS-MES-buffer and acetone (-20°C). For collection of ionically cell wall-bound POD, cell walls were incubated for 2 h in 1M NaCl in 50 mM MES-TRIS (pH 7) and for 1 h in 1 M LiCl in 50 mM MES-TRIS (pH 7) at 4°C. Cells were washed again in aqua dest. and incubated with an enzyme mixture containing 0.01% pectolyase, 0.1% cellulase and 1% BSA in 50 mM TRIS-MES (pH 5.5) for 1 h at 26°C to extract covalently bound POD from the cell wall.

Concentrations of AA and DHA in the AWF and extracted from the leaf tissue were measured according to Takahama and Oniki (1992). The concentrations of AA and DHA were calculated from the absorption of the sample in 100 mM Na_2HPO4 (pH 6.8) at λ=265 nm (i) without any addition, (ii) with addition of ascorbate oxidase (1 unit/100µl), (iii) and with addition of 10 mM DL-Dithiothreitol.

Peroxidase (POD, EC 1.11.1.7) activities in the AWF and extracted from the leaf tissue were determined spectrophotometrically at λ=470 nm by following H_2O_2-depending oxidation of guaiacol. Samples were mixed with guaiacol solution (20 mM guaiacol in 10 mM Na_2HPO_4 buffer (pH 6)) and 0.03% H_2O_2.

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2) were assayed according to Polle et al. (1990). The assay mixture for MDHAR determination comprised 80 mM K_2HPO_4 (pH 7.8), 200 µM NADH, 0.5 mM ascorbic acid (AA) and ascorbate oxidase (AAO, 1 mg ml^{-1}, Sigma Aldrich). The decomposition of NADH was followed by the decline in absorbance at λ=340 nm for 2 min. Changes in absorption without AA and AAO were taken into consideration.

Glutathione reductase (GR) was assayed by measuring the degradation of NADPH at λ=340 nm. According to Polle et al. (1990) measurements were carried out in 50 mM HEPES buffer (pH 8.0) containing 0.5 EDTA, 0.5 mM oxidized glutathione (GSSG) and 0.25 mM NADPH. Changes in absorption without GSSG were deduced.

To identify water-soluble apoplastic isoenzymes of POD the apoplastic washing fluid was lyophilised and resuspended in sample buffer. Separation was performed with disc electrophoresis using a 4% stacking and 10% separating acrylamid-gel. Sample buffer and polyacrylamid gels were prepared without SDS and reducing agents like DTT and mercaptoethanol to maintain native enzyme conformation. Gels were loaded with approximately 100 µg protein. After 60 min running time at 150 V at 4°C, gels were stained with a guaiacol-H_2O_2 mixture containing 20 mM guaiacol in 10 mM Na_2HPO_4 buffer, pH 6.0 and 0.01% H_2O_2.

For detection of protein contents in the AWF a fluorometric method was used according to Böhlen et al. (1973).

Manganese in the bulk-leaf tissue was determined after dry ashing and dissolving the ash in 6 M HCl with 1.5% (w/v) hydroxylammonium chloride, in 1/10 diluted solution by ICP-OES (Spektro Flame).

Statistical analysis was carried out using SAS Release 8.0. Coefficients of determination from regression analysis and results from analysis of variance are given according to their level of significance as ***, **, * or $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively.

RESULTS

The intensity of first symptoms of Mn toxicity was closely correlated with the bulk-leaf Mn content (Fig. 1). The Mn-sensitive cv. TVu 91 showed a higher density of brown spots than the Mn-tolerant cv. TVu 1987 at given elevated levels of Mn in the leaf tissue.

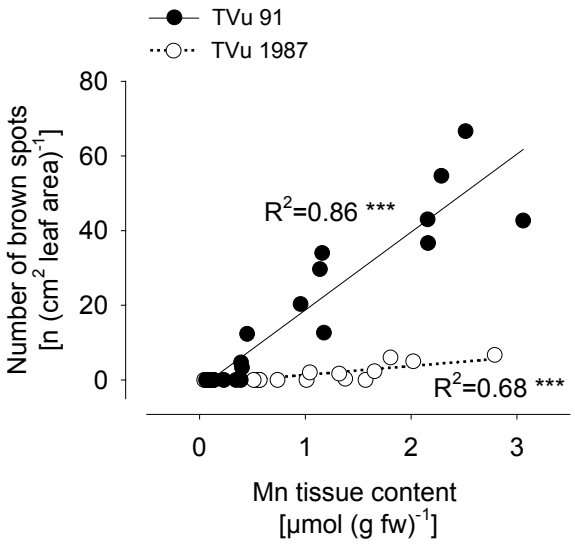

Figure 1 Relationship between leaf-tissue Mn content and density of brown spots as affected by cultivar. Plants of cvs. TVu 91 and TVu 1987 precultured for 15 days were treated with 50 and 100 µM Mn for 6 days, whereas control plants received 0.2 µM Mn continuously.

The activities of cytoplasmic, water-soluble apoplastic and ionically bound cell-wall POD were generally significantly higher in leaves of cv. TVu 91 than in cv. TVu 1987 (Fig. 2). The POD activity in the apoplastic washing fluid was much higher than in the other leaf fractions and was significantly enhanced with increasing bulk-leaf Mn contents in the Mn-sensitive cultivar but not in the Mn-tolerant cv. TVu 1987.

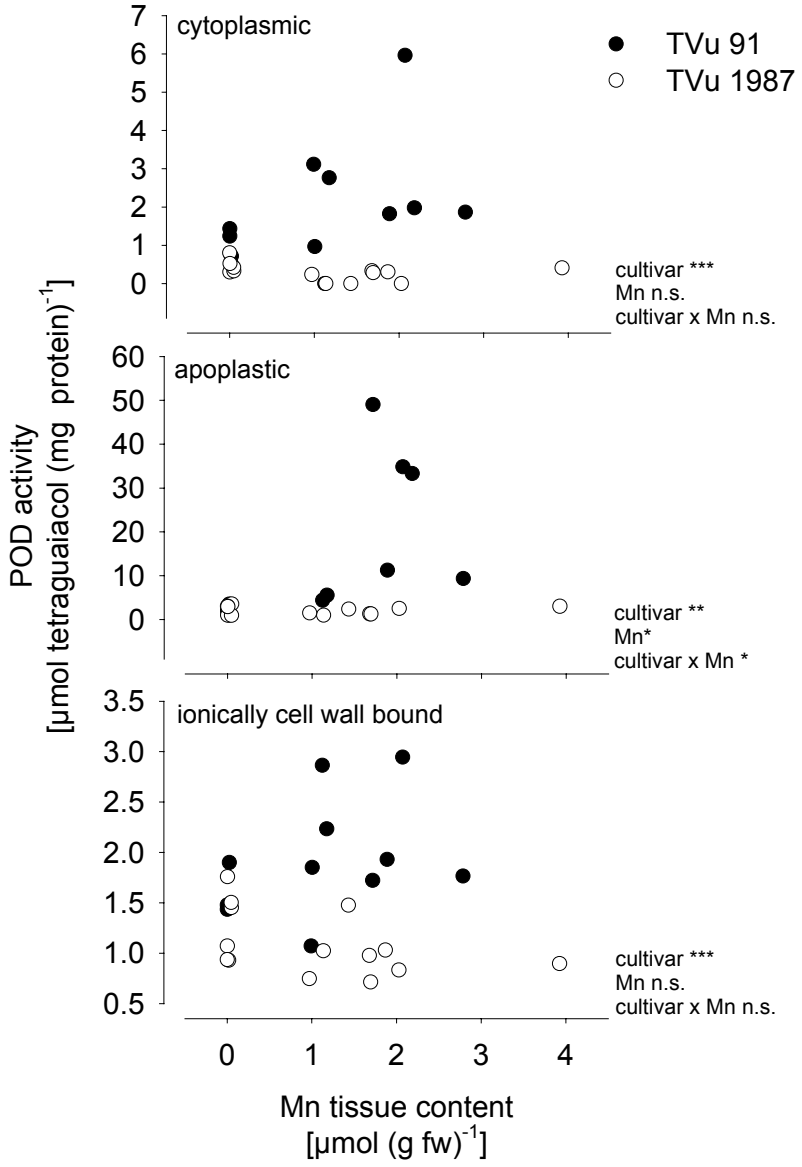

Figure 2 Relationships between leaf-tissue Mn contents and activities of cytoplasmic, apoplastic water-soluble and ionically cell wall-bound POD. Plants of cvs. TVu 91 (Mn-sensitive) and TVu 1987 (Mn-tolerant) precultured for 15 days were treated with 50 and 100 µM Mn for 6 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * or $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively.

To specify the effect of Mn on the POD activities of different fractions, the POD activities of cv. TVu 91 were calculated based on protein content as well as on FW. The supply of

100 µM Mn strongly enhanced the total combined POD activities of all fractions (Table 1), independent of the basis of reference.

Table 1 Effect of Mn supply on the activities of apoplastic water-soluble, ionically cell wall-bound, covalently cell wall-bound and cytoplasmic POD calculated on the basis of protein content and leaf fresh weight. Plants of cv. TVu 91 (Mn-sensitive) precultured for 15 days were treated with 100 µM Mn for 6 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * or $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively.

cv. TVu 91

	Peroxidase activity [µmol tetraguaiacol (mg protein)$^{-1}$]					Peroxidase activity [µmol tetraguaiacol (g fw)$^{-1}$]				
Mn supply [µ*M*]	0.2		100			0.2		100		
Apoplastic										
water soluble	4.81	(64)	34.53	(85)	*	1.48	(21)	13.28	(58)	*
ionically bound	1.64	(22)	2.15	(5)	n.s.	2.54	(35)	3.37	(15)	n.s.
covalently bound	n.d.		n.d.			0.1	(1)	0.19	(1)	n.s.
Cytoplasmic	1.12	(15)	3.92	(10)	n.s.	3.08	(43)	6.09	(27)	n.s.
Total	7.57		40.60			7.2		22.93		

( ) in percent of total
n.d. not determined

This was only due to a significant increase of the apoplastic water-soluble POD. Electrophoresis of apoplastic washing fluid of control plants (optimum Mn supply) revealed the presence of a similar pattern of several water-soluble POD isoenzymes (Fig. 3) in both cultivars. At a toxic Mn supply of 100 µM, the activities of these isoenzymes increased much more in the Mn-sensitive than in the Mn-tolerant cultivar. Also, new bands became visible indicating the expression of additional POD isoenzymes.

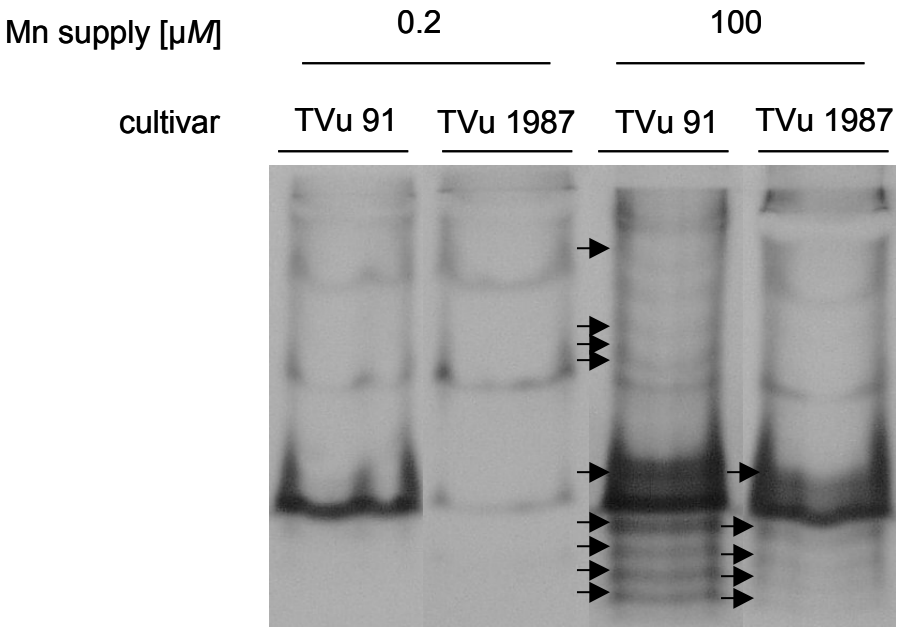

Figure 3 Effect of Mn supply on the quantity and intensity of the POD isoenzyme pattern revealed by disc electrophoresis. Apoplastic washing fluid extracted from cv. TVu 91 (Mn-sensitive) and TVu 1987 (Mn-tolerant) was concentrated and separated using a 4% stacking and 10% separating gel under native conditions. Peroxidases were stained with a guaiacol-H_2O_2 test-mixture. Activated or released POD into the apoplast due to Mn treatment are marked by arrows.

Bulk-leaf Mn contents rapidly increased after increasing the Mn supply from 0.2 to 50 µM in both cultivars (Fig. 4) reaching a plateau after 3-4 days. First visible Mn toxicity symptoms appeared on day 3 in the Mn-sensitive cultivar whereas in the Mn-tolerant cultivar it took 8 days for brown spots to appear. In the Mn-sensitive cv. TVu 91 the density of brown spots increased from day 3 to 6 without a significant change of the Mn contents. The POD activity showed a very similar pattern as the density of brown spots: after 3 days of elevated Mn treatment, POD was significantly enhanced in the Mn-sensitive cultivar and then steadily increased whereas in cv. TVu 1987 no increase was observed.

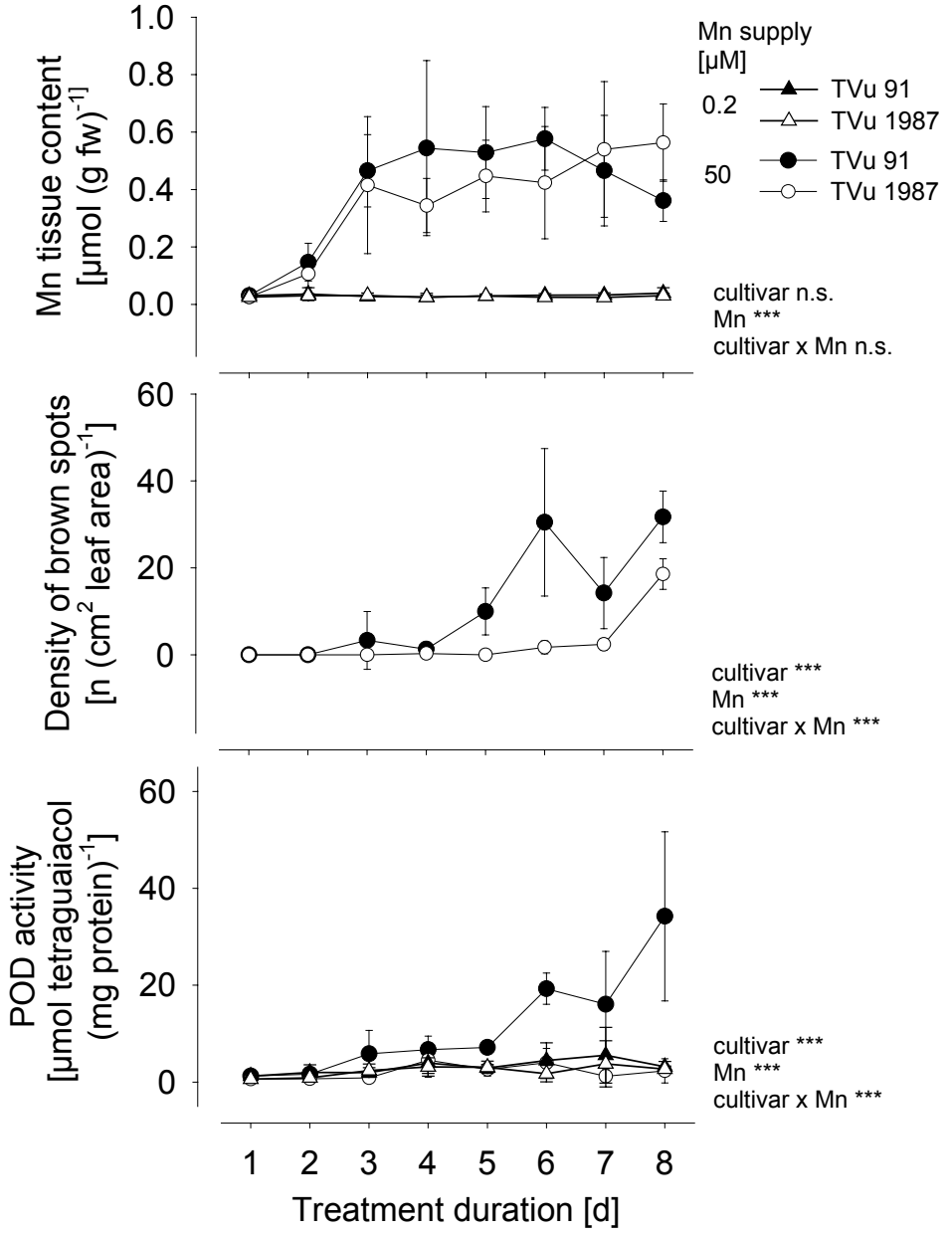

Figure 4 Effect of excess-Mn treatment-duration on leaf-tissue Mn contents, density of brown spots and POD activity in the apoplastic washing fluid (AWF) as affected by cowpea cultivar. After 15 days of preculture, plants of cvs. TVu 91 (Mn-sensitive) and TVu 1987 (Mn-tolerant) were treated with 50 µM Mn, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * or $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively.

The concentration of AA and the ratio of AA/(AA+DHA) in the AWF of cv. TVu 91 but not of cv. TVu 1987 decreased significantly with increasing bulk-tissue Mn content (Fig. 5). The AA content and the ratio of AA/(AA+DHA) in the bulk-leaf tissue were similarly dependent on the Mn tissue content (Fig. 6).

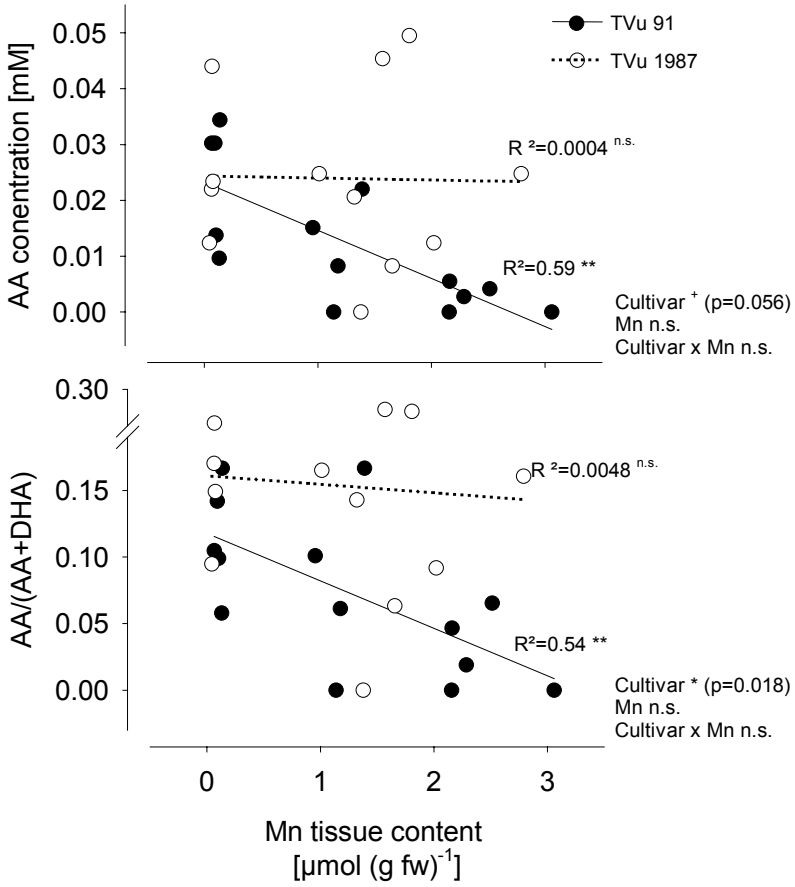

Figure 5 Relationships between leaf-tissue Mn content and the concentration of free AA or the AA/(AA+DHA) ratio in the apoplastic washing fluids (AWF) of cowpea cvs. TVu 91 (Mn-sensitive) and TVu 1987 (Mn-tolerant). Plants precultured for 15 days were treated with 50 and 100 µM Mn for 6 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * or $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively

Therefore, the AA concentration in the AWF, the AA content in the leaf tissue and the ratios of apoplastic and leaf tissue AA/(AA+DHA) were significantly lower in cv. TVu 91 than in cv. TVu 1987 at elevated leaf-tissue Mn contents.

The activities of the AA-regenerating enzymes MDHAR and GR (DHAR was not detectable) were significantly enhanced at higher Mn tissue contents particularly in the Mn-tolerant cv. TVu 1987 (Fig. 7).

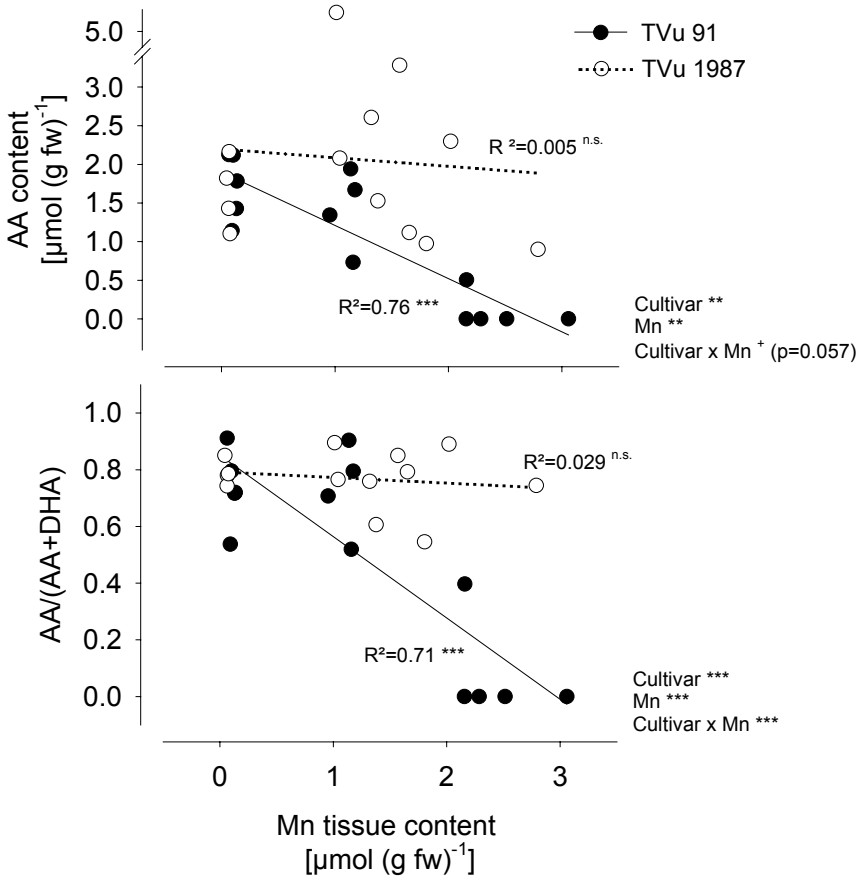

Figure 6 Relationships between leaf-tissue Mn content and the content of free AA or the AA/(AA+DHA) ratio in the leaf tissues of cowpea cvs. TVu 91 (Mn-sensitive) and TVu 1987 (Mn-tolerant). Plants precultured for 15 days were treated with 50 and 100 µM Mn for 6 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * or $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively.

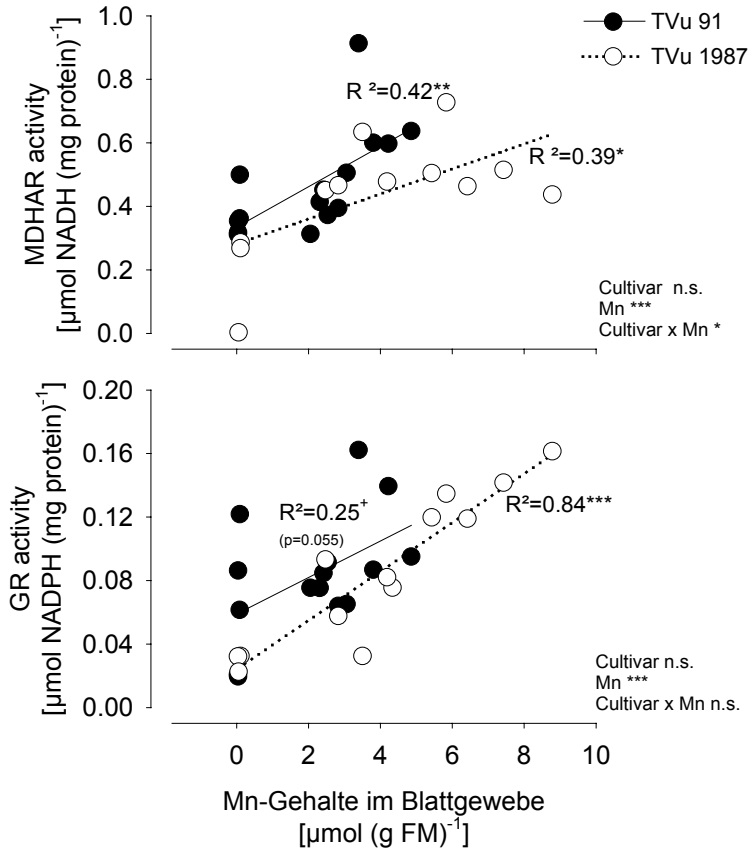

Figure 7 Relationships between leaf-tissue Mn content and activities of the ascorbate regenerating enzymes MDHAR and GR extracted from the leaf tissues of cowpea cvs. TVu 91 (Mn-sensitive) and TVu 1987 (Mn-tolerant). Plants precultured for 15 days were treated with 50 and 100 µM Mn for 6 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * or $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively.

DISCUSSION

In agreement with Horst (1980), cv. TVu 1987 proved to be more Mn-tolerant than cv. TVu 91. This is related to its ability to tolerate high Mn contents in the leaf tissue. The effect of the bulk-leaf Mn content on the activity of the examined types of POD from leaves of cv. TVu 91 and the significant difference between the cultivars TVu 91 and TVu 1987 (Fig. 2) clearly support the hypothesis (Horst et al., 1999) that in cowpea, Mn toxicity and Mn toxicity-symptoms are mediated by a H_2O_2-POD system. The calculation of guaiacol-POD activities on the basis of protein content and fresh weight show that the POD in the AWF was affected most strongly by the Mn treatment. The deposition of brown substances in the cell wall (Wissemeier and Horst, 1992) linked with a strong activation of apoplastic water-soluble POD suggest that the apoplast is the main site for the expression of Mn toxicity. The simultaneous browning of the leaves and increasing activity

of POD after 3 days of treatment support the idea of a close relation between Mn-toxicity and a POD system in the apoplast. The quantitative and qualitative changes in the expression of POD isoenzymes owing to excess Mn treatment and between the cultivars are consistent with the POD activities measured in the AWF. Mn treatment-enhanced POD activity was also found in Mn-sensitive cultivars of soybean (Leidi et al., 1989). This was accompanied by specific differences in POD isoenzyme patterns at very high Mn levels. The simultaneous induction of POD and H_2O_2 formation by Mn treatment (Horst et al., 1999) is indicative for a basic stress response (Castillo, 1986; Siegel and Siegel, 1986). It remains unclear whether the increase in POD activity is due to a direct stimulation of this enzyme by Mn, or indirectly via an Mn-induced oxidative burst as has been proposed as a response to pathogen infection (Wojtaszek, 1997). Kenten and Mann (1950) found a close relation between the oxidation of Mn by a horseradish extract containing POD in the presence of H_2O_2 and monophenols, suggesting that intermediate products of phenol oxidation by a H_2O_2-POD system lead to Mn^{II} oxidation. It was also shown that oxidation of Mn *in vitro* can be induced by an enzyme system producing H_2O_2 coupled with peroxidase (Kenten and Mann, 1952).

The oxidation of Mn and phenols could not only lead to the deposition of Mn oxides (Kenten and Mann, 1956) and oxidized phenols in the cell wall of cowpea (Wissemeier and Horst, 1992) but also to plant injury by oxidative stress due to the reaction of phenoxyradicals and reactive Mn^{III} with several apoplastic components such as the plasmamembrane (lipidperoxidation).

Different enzymatic mechanisms for H_2O_2 generation in the apoplast are currently being discussed. The formation of H_2O_2 needed for lignification has been attributed to a NADPH or NADH-dependent cell wall-bound peroxidase (Elstner and Heupel, 1976). The oxidation of NADH with subsequent formation of H_2O_2 was strongly accelerated by Mn^{II} and various monophenols in a complex pathway (Gross et al., 1977; Halliwell, 1978). Cell-wall peroxidases were classified in acidic and basic isoforms with different pH optima and favored substrates (Sato et al., 1995; Mehlhorn, et al., 1996; Otter and Polle, 1997) according to their involvement in the production and consumption of H_2O_2 during lignification (Mäder et al., 1980). It was suggested that PODs are polyfunctional enzymes which, depending on the pH in the apoplast, catalyse the oxidative polymerization of cinnamyl alcohols and NADH-dependent H_2O_2 formation, respectively (Pedreño et al., 1989; Pedreño et al. 1995). Under the condition of Mn toxicity, Mn^{II} could act as a powerful inductor of POD-catalyzed H_2O_2 production in the apoplast, which leads in turn

to an activation of the H_2O_2-POD system with the subsequent formation of brown spots in the cell wall. The similar activity pattern obtained from cv. TVu 91 and cv. TVu 1987 at low Mn supply suggests that the same POD isoenzymes were active in the apoplast in both cultivars. But considering the wide range of POD-isoenzymes in cellular localization and function, considerable differences between the cultivars cv. TVu 91 and cv. TVu 1987 concerning their POD-isoenzyme pattern in the apoplast cannot be excluded.

The role of ascorbic acid (AA) as an effective scavenger for oxidative compounds is well known (Polle and Rennenberg, 1993) and the regulatory effect of ascorbic acid on the peroxidase-catalysed oxidation of phenols in the apoplast has been reported (Takahama and Oniki, 1992; Takahama, 1993; Sánchez et al., 1997). Takahama (1993) suggested that ascorbic acid acts as a secondary electron donator by reducing phenoxyradicals resulting in a complete inhibition of the radical chain reaction. The decrease of ascorbic acid in the apoplast and leaf tissue of the sensitive cv. TVu 91 by Mn treatment (Figs. 5 and 6) indicates that Mn toxicity is mediated by the formation of oxidative compounds. Figure 8 schematically shows the proposed linkage between peroxidase-mediated oxidation of Mn^{II} and phenols, and the role and regeneration of ascorbic acid involved in the scavenging of reactive phenolic intermediates and Mn^{III}. The effectiveness of the ascorbate regenerating system involving the enzymes monodehydroascorbic acid reductase (MDHAR), dehydroascorbic acid reductase (DHAR) and glutathione reductase (GR), and the maintenance of the transport of AA and DHA across the plasma membrane (see Noctor and Foyer, 1998; Horemans et al., 2000; Smirnoff, 2000) may contribute to control oxidative plant injury. While DHAR was not detectable, a close relationship was found between Mn tissue content and the activities of the regenerating enzymes MDHAR and GR (Fig. 7). The Mn-tolerant cv. TVu 1987 showed an increase in enzyme activities, particularly GR, without decreasing apoplastic AA concentration and leaf tissue content. This indicates that in this cultivar Mn triggers the AA regeneration system thus maintaining a high AA level in the apoplast. In the Mn-sensitive cultivar this regeneration system appeared to be insufficiently effective to balance AA oxidation.

It remains unclear if genotypic differences in Mn tolerance are due to the antioxidative capacity via an ascorbic acid system in the apoplast or in the cytoplasm. Changes of AA in the leaf tissue and a higher depletion of AA in the sensitive cultivar after Mn treatment were found in common bean (Gonzáles et al., 1998), whereas an effect of Mn on the AA regenerating enzyme GR was not observed. However, plants increase several components

of the antioxidative system in response to stresses, whereas apparently a balanced increase of the antioxidative capacity is required for stress tolerance (Polle and Rennenberg, 1993). In conclusion, although the mechanisms of Mn toxicity and tolerance could not been fully elucidated, the results provide circumstantial evidence that ascorbate and peroxidases are involved in the expression of Mn toxicity and genotypic Mn tolerance.

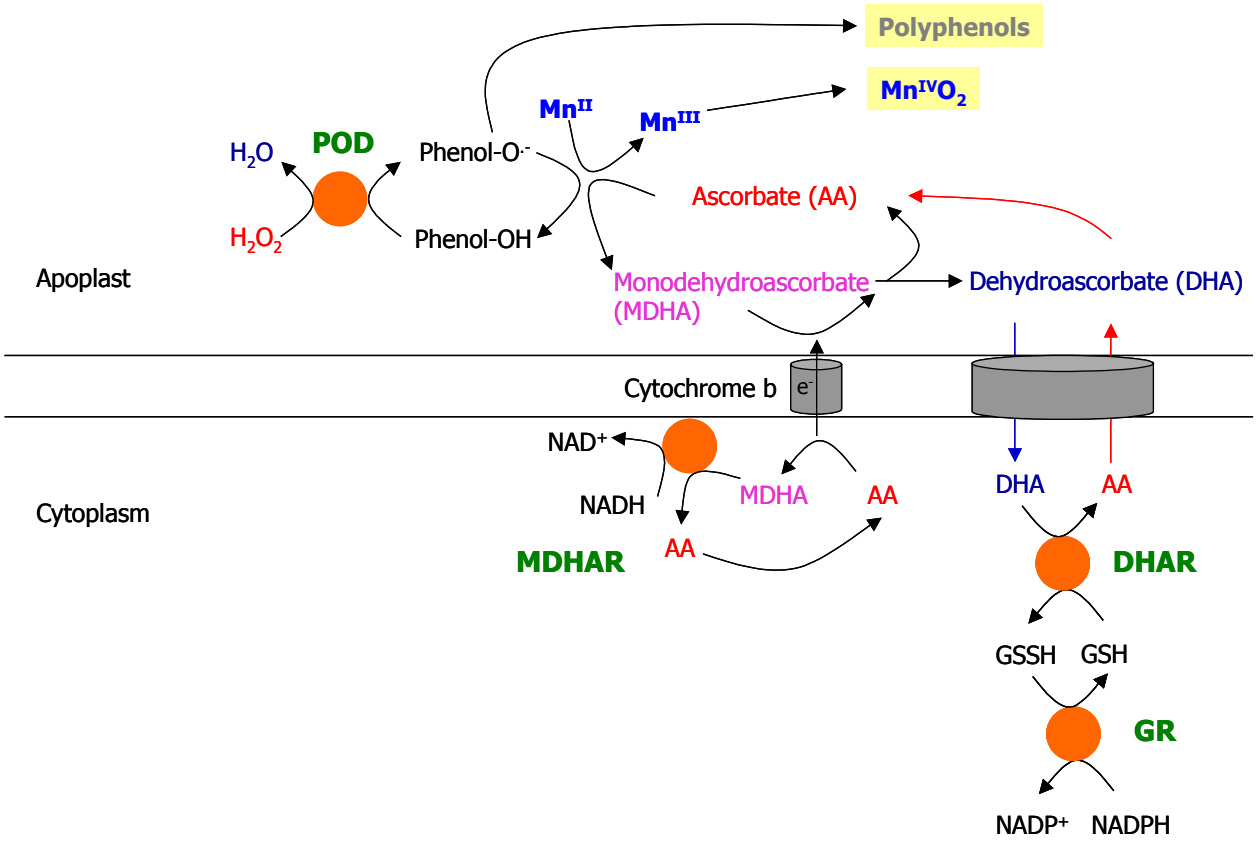

Figure 8 Proposed mechanisms in the apoplast of cowpea induced by excessive Mn treatment. Phenol- und Mn-oxidation are linked together via a H_2O_2-POD system in the apoplast. Mn^{III} is formed by the reaction of Mn^{II} with phenoxyradicals. Ascorbic acid scavenges reactive phenolic intermediates and Mn^{III}. Oxidized ascorbate is reduced (i) directly in the apoplast by electron transfer via a membrane located cytochrome b, which is associated with MDHAR, or (ii) by a regeneration cycle in the cytoplasm including DHAR and GR. Based on Noctor and Foyer, 1998; Horemans et al., 2000; Smirnoff, 2000.

CHAPTER 2:

EFFECT OF MANGANESE TOXICITY ON THE PROTEOME OF THE LEAF APOPLAST IN COWPEA

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ABSTRACT

Excess manganese (Mn) supply causes formation of visible brown depositions in the cell wall of leaves of cowpea (*Vigna unguiculata*), which consist of oxidized Mn and oxidized phenols. Because oxidation of Mn and phenolic compounds in the leaf apoplast was proposed to be catalyzed by apoplastic peroxidases (PODs), induction of these enzymes by Mn excess was investigated. POD activity increased upon prolonged Mn treatment in the leaf tissue. Simultaneously, a significant increase in the concentration of soluble apoplastic proteins in "apoplastic washing fluid" (AWF) was observed. The identity of the released proteins was systematically characterized by analysis of the apoplast proteome using two-dimensional gel electrophoresis and liquid-chromatography tandem mass spectrometry. Some of the identified proteins exhibit sequence identity to acidic peroxidases from other plants. Several other proteins show homologies to pathogenesis-related proteins, e.g. glucanase, chitinase and thaumatin-like proteins. Because pathogenesis-related-like proteins are known to be induced by various other abiotic and biotic stresses, a specific physiological role of these proteins in response to excess Mn supply remains to be established. The specific role of apoplastic PODs in the response of plants to Mn stress is discussed.

INTRODUCTION

For a wide range of plant species, formation of brown spots is part of a characteristic development of Mn toxicity symptoms in older leaves. The subsequent development of chlorosis and necrosis and finally leaf shedding occurs before a reduction in vegetative growth on the whole plant level (Horst, 1988; El-Jaoul and Cox, 1998). Analysis of the Mn-induced formation of brown spots revealed the presence of oxidized Mn and oxidized phenols, especially in the cell wall of the epidermis layer (Horiguchi, 1987; Wissemeier and Horst, 1992). The formation of visible Mn-toxicity symptoms is accompanied by the spatial formation of callose in the area of brown spots (Wissemeier and Horst, 1987). The physiological role of callose formation in response to toxic Mn levels in the tissue is unknown, but its detection serves as an additional sensitive parameter for Mn induced injury of the leaf tissue. In cowpea (*Vigna unguiculata*), the leaf apoplast has been proposed to be the most important compartment for the defense of Mn stress (Horst et al., 1999). The oxidation of Mn^{II} by a H_2O_2-consuming peroxidase (POD) has been proposed

to be the key reaction leading to Mn-toxicity symptoms, probably accompanied by the formation of reactive intermediate compounds like phenoxy radicals and Mn^{III} (Horst, 1988). This hypothesis is supported by a close relationship between the Mn-induced formation of brown spots, activation of constitutive apoplastic POD, and Mn-induced release of POD into the apoplast (Fecht-Christoffers et al., 2003). Among the existing information about the physiological functions of POD in plant tissue, its activation in response to a broad range of biotic and abiotic factors plays a particularly important role (Greppin et al., 1986; Obinger et al., 1996). POD activity is often used as a physiological marker for plant-stress response as part of a complex cascade of reactions with an apparent lack of specificity. However, some specific relationships between horseradish POD and the oxidation of Mn^{II} and phenolic compounds in the apoplast were reported by Kenten and Mann (1950). In particular, the stimulating effect of Mn on apoplastic H_2O_2-producing PODs (Halliwell, 1978) provides evidence of a specific role of Mn on the functionality of PODs in the leaf apoplast.

To get a better understanding of the specific involvement of POD and other proteins in the response of cowpea plants to Mn excess, observations on changes of POD activities and further physiological changes in the apoplast were investigated at different stages of Mn toxicity. Furthermore, two-dimensional (2D) gel electrophoresis techniques were used for the separation of apoplastic water-soluble proteins followed by a systematic identification of proteins using nanoscale capillary liquid chromatography-tandem mass spectrometry (nano LC-MS/MS). Most of the identified proteins exhibit sequence similarity to previously characterized proteins from other organisms, which partially are known to play roles in stress defense. These proteins represent new tracks for the investigation of Mn stress in higher plants.

MATERIALS AND METHODS

Plant material

Cowpea (*Vigna unguiculata* [L.] Walp. cvs TVu 91 and TVu 1987) was grown hydroponically in a growth chamber under controlled environmental conditions at 30/25°C day/night temperature, 75%±5 % relative humidity, and a photon flux density of 270 µmol $m^{-1}s^{-1}$ photosynthetic active radiation at mid-plant height during a 16-h photoperiod. After germination in 1 mM $CaSO_4$, seedlings were transferred to a constantly aerated nutrient solution. After preculture, the $MnSO_4$ concentration in the nutrient solution was increased,

whereas control plants received 0.2 µM Mn continuously. The nutrient solution was changed two to three times a week to avoid nutrient deficiencies.

Quantification of toxicity symptoms

For the quantification of Mn toxicity symptoms, the density of brown spots was counted on a 1 cm^2 area at the base, middle and tip on the upper side of the trifoliate leaf.

Extraction of water soluble proteins from the leaf apoplast

AWF was extracted by a vacuum infiltration/centrifugation technique. Leaves were infiltrated with water by reducing the pressure to 35 hPa followed by a slow relaxation for 2 min. The AWF was recovered by centrifugation at 1,324*g* for 5 min at room temperature.

Mineral analysis

Mn in the bulk-leaf tissue was determined after dry ashing (480°C, 8h) and dissolving the ash in 6 M HCl with 1.5% (w/v) hydroxylammonium chloride and diluted 1:10 with water. AWF was diluted 1:10, whereas HCl and hydroxylammonium chloride were added to give final concentration of 0.6 M HCl and 0.15% hydroxylammoniumchloride. Measurements were carried out by optical emission spectroscopy, inductively-coupled plasma (Spectro Analytical Instruments GmbH, Kleve, Germany)

Callose extraction and detection

For the measurement of callose formation, four leaf discs (150 mg fresh weight) were cut out of the leaf and fixed in ethanol. After 3 d ethanol was replaced by demineralized water and incubated over night. Leaf discs were homogenized in 1 mL of 1M NaOH, and homogenates were incubated for 15 min at 80°C in a water bath. After centrifugation at 13,000 g for 5 min, 100 µL of the supernatant was mixed with 600 µL of the anilin mix (0.59 M Gly buffer [pH 9.5], 0.21 M HCl, and 0.04% [w/v] anilin blue) and incubated in a 50°C water bath for 20 min. After cooling the samples down to room temperature, the callose concentration was measured by detecting the fluorescence at excitation wavelength/bandpath of 400nm/30nm and emission wavelength/bandpath of 485nm/40nm with a microplate reader (BioTek-Instruments, Germany). Control measurements were done with Gly-HCl-buffer solution without anilin blue. For the calculation, the molar extinction coefficient ε=11.32 $(mg\ L^{-1})^{-1}$ was used.

POD activity in the AWF

Guaiacol-POD activities in the AWF were determined spectrophotometrically at λ=470 nm by following the H_2O_2-depending oxidation of guaiacol. Samples were mixed with guaiacol solution (20 mM guaiacol in 10 mM Na_2HPO_4 buffer [pH 6] and 0.03% [w/w] H_2O_2).

NADH-POD activities in the AWF were determined spectrophotometrically at λ=340 nm by following decline of NADH. Samples were mixed with 0.6 mM NADH, 1.6 mM p-coumaric acid and 16 mM $MnCl_2$ in 100 mM NaAc buffer (pH 5).

Detection of total protein in the AWF

The protein concentration in the AWF was measured according to Bradford (1976).

Extraction of proteins from the AWF

For protein separation by electrophoresis under native conditions, the proteins of the AWF were purified at 4°C by using centrifugal concentrators with a molecular mass cut off at 5kD (Vivaspin 6, Vivascience, Hannover, Germany). Run conditions were used according to the manufacturer`s instructions.

For the separation by IEF, proteins were extracted by phenol and precipitated by acetate/methanol. The volume of the AWF was reduced by lyophilisation. The sample was mixed with extraction buffer (700 mM saccharose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, and 2% [v/v] mercaptoethanol), and after incubation for 10 min on ice, an equal volume of phenol (saturated solution, pH 6.6/7.9, [TRIS], FA Amresco, Solon, OH) was added and shaken for 30 min. The aqueous and organic phases were separated by centrifugation for 10 min at 5,000 g and 4°C. The phenolic phase was re-extracted with extraction buffer and centrifugated once more. The phenol phase was combined with 5 volumes of 0.1 M ammonium acetate in methanol and incubated for approximately 14 h at –20°C. After centrifugation at 20,000 g for 5 min at 4°C, precipitated proteins were washed three times with ammonium acetate in methanol and finally with acetone. The protein samples were air dried and resuspended immediately before electrophoresis.

2D BN/SDS-PAGE

A detailed protocol for 2D BN/SDS-PAGE was published by Schägger (2001). Protein samples were combined with a Coomassie-blue solution (5% [w/v] Serva Blue G, 750 mM

aminocaproic acid). Samples were loaded onto a native acrylamid gel with a 4% (w/v) acrylamide stacking gel and a 12% to 20% (w/v) gradient separation gel. After electrophoresis the gel was soaked in 20 mM guaiacol and 0.03% (w/w) H_2O_2 to detect PODs in the gel. Gel slices without incubation in peroxidase staining solution were used for the second electrophoresis dimension. For this application, gels were first incubated in 1% (w/v) SDS/1% (v/v) mercaptoethanol for 30 min. Separation gels for the second gel dimension had 10% or 16.5% (w/v) acrylamide, whereas the sample gels always had 10% (w/v) acrylamide. Gel electrophoresis was carried out in Protean II Xi and XL cells (gel dimension 20 x 18 cm) from Bio-Rad (Munich).

2D IEF/SDS-PAGE

For separation of proteins by their pI, the IPGphor system (Amersham Biosciences, Uppsala) availing Immobiline DryStrip gels (18 cm) with a nonlinear pH gradient (pH 3-10) was used. Proteins were resuspended in demineralized water and supplemented with a rehydration solution (8M urea, 2% [w/v] CHAPS, 0.5% [v/v] carrier ampholyte mixture [IPG buffer; Amersham Biosciences], and a trace of bromphenol blue). Focussing conditions were used according to Werhahn and Braun (2002). Afterwards, the Immobiline DryStrip gels were incubated with equilibration solution (50 mM Tris-Cl [pH 8.8], 6 M urea, 30% [v/v] glycerin, 2% [w/v] SDS, and bromphenol blue) supplemented with (a) 1% (w/v) dithiothreitol and (b) 2.5% (v/w) iodoacetamide each for 15 min, respectively. DryStrips were placed horizontally on a Tricine SDS-PAGE. The second dimension electrophoresis was carried out according to Schägger and von Jagow (1987).

Staining

2D gels and the 1D BN-gels were stained with colloidal Coomassie-blue according to Neuhoff et al. (1985, 1990).

Protein preparation and identification by nano LC-MS/MS

After staining with colloidal Coomassie-blue single proteins were cut out and transferred in Eppendorf vessel. Exised slices rinsing and reduction/alkylation steps were performed by the Massprep (Micromass, Manchester, UK) robot. Each piece of gel was washed with 100 µl of 25 mM NH_4HCO_3 and dehydrated with 100 µl acetonitrile (ACN). This operation was repeated twice. Reduction was achieved by 1 hour treatment with 10 mM DTT at

room temperature. Alkylation reaction was performed by 25 mM iodacetamide for 45 min at room temperature and protected from light. Finally, gel spots were washed 3 times for 5 min again alternately with 25 mM ammonium carbonate and ACN. Gel pieces were completely dried before tryptic digestion and rehydrated by trypsin addition. About three volumes trypsin freshly diluted (brackets around 12.5 ng/µl in 25 mM NH_4HCO_3 buffer) were added to the sample. Digestion was performed overnight. The gel pieces were centrifuged and 60 µl of 35% (v/v) H_2O/60% (v/v) ACN/5% (v/v) formic acid (HCOOH) were added to extract peptides. The mixture was sonicated for 30 min. The supernatant was recovered and the operation was repeated once. For nano LC-MS/MS, the supernatants were transferred into a 96 wells plate and the peptide extraction volume was reduced to 10 µl by evaporation in order to concentrate the peptides and to remove the ACN from the sample before injected in the HPLC system. Nanoscale capillary liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis of the digested proteins were performed using an CapLC capillary Liquid Chromatography system (Micromass, Manchester, UK) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF II, Micromass). 6.4 µl of sample were loaded and concentrated onto a C18 PepMap precolumn (LC Packing) under a 30 µl/min flow rate and flushed for 3 min with 0.1% (v/v) ACN before gradient started to elute the peptides straight to the following separative column. Chromatographic separations were then performed on a reversed-phase capillary column (Pepmap C18, 75µm i.d., 15 cm length, LC Packings) under a 200 µL/min flow rate generated by the CapLC delivering a flow rate of 4.5 µl/min splitted right after the precolumn. The gradient profile used consisted of a linear gradient from 95% A (H_2O / 0.1 % [v/v] HCOOH) to 60 % B (ACN / 0.1 % [v/v] HCOOH) in 35 min followed by a linear gradient to 95% B in 1 min. Mass data acquisitions were piloted by MassLynx software (Micromass, Manchester, UK).

When MS/MS required, automatic switching between MS and MS/MS modes was used and the internal parameters of Q-TOF II were set as follows. The electrospray capillary voltage was set to 3.5 kV, the cone voltage set to 40 V, and the source temperature set to 120°C. The MS survey scan was m/z 300-1500 with a scan time of 1s and a interscan time of 0.1s. When the intensity of a peak rose above a threshold of 10 counts, tandem mass spectra were acquired. Normalized collision energies for peptide fragmentation was set using the charge-state recognition files for +2 and +3 of the 3 more intense ion parents. The scan range for MS/MS acquisition was from m/z 50 to 1500 with a scan time of 1 s and an interscan time of 0.1s. MS/MS acquisition switched back to MS when threshold

reached 2 or after 10 sec acquisition duration. Fragmentation was performed using argon as the collision gas and with a collision energy profile optimised for various mass ranges of ion precursors. Mass data collected during a LC-MS/MS analysis were processed and converted into a .PKL file to be submitted to the Global server 1.1 and Mascot search engines. Global server 1.1 and Mascot searches were first performed against SwissProt Data Bank with a tolerance on mass measurement of 0.25 Da in MS mode and 0.5 Da in MS/MS mode without any pI and MW restrictions but variable modifications were taken into account, like methionine oxidation. The peptide mass error was limited to 50 ppm. For confirmation, each spectra were loaded onto the Peptide Sequencing software (BioLynx, Micromass, UK) and the sequences re-processed manually before submitted to a Blast search (NCBI) without any taxonomy restriction. Most obtained sequences represent complete tryptic peptides. However, the sequences of some other peptides could only be partially determined by MS/MS.

Statistical analysis

Statistical analysis was carried out using SAS Release v8.0 (SAS Institute, Cary, NC). Coefficients of determination from regression analysis and results from analysis of variance are given according to their level of significance as ***, **, * and $^{+}$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively. Different letters are significantly different at $p < 0.05$ (Tukey).

RESULTS

Effect of Mn treatment duration on Mn uptake, activity of apoplastic guaiacol- and NADH-POD, protein concentration in the "apoplastic washing fluid" (AWF), and callose formation

Mn is readily taken up by cowpea plants and transported to leaves. The Mn tissue content increased exponentially 40-fold during 6 days of treatment with 50 µM Mn (Fig. 1A). The related increase of the Mn concentration in the AWF followed a saturation curve (Fig. 1B) with a significant increase already after one day of Mn treatment. The ratio of water-soluble Mn in the AWF and total Mn of cowpea leaves was about 0.3% (Fig. 1C). After one day of Mn treatment, it increased significantly to 1.2%. After 3 days of Mn treatment, it decreased to the initial ratio, which seems to represent an equilibrium with the total Mn in the tissue.

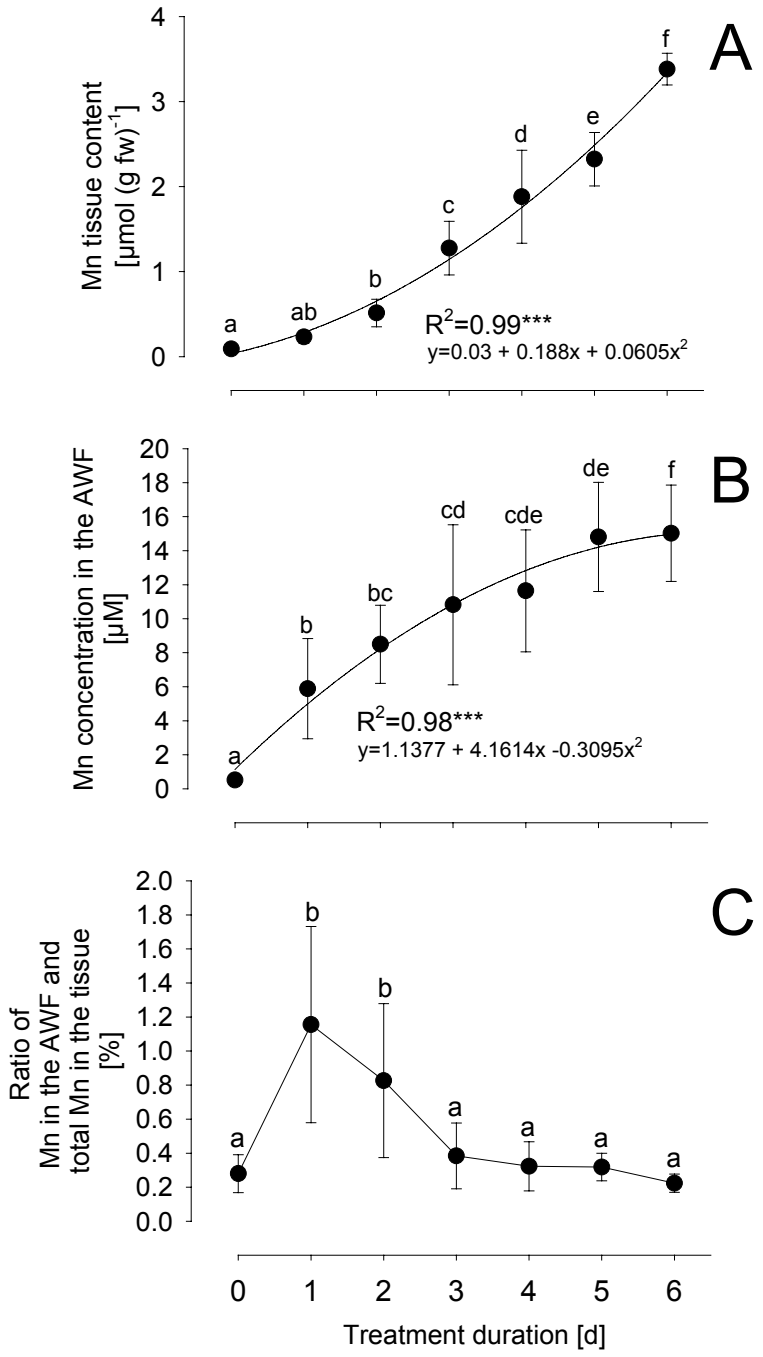

Figure 1 Effect of treatment duration on (A) the total Mn content in the leaf tissue, (B) the Mn concentration in the apoplastic washing fluid of leaves, and (C) the ratio of water-soluble Mn in the apoplast to total Mn in the leaf tissue. Plants of cowpea were treated with 50 µM Mn, whereas control plants received 0.2 µM Mn, continuously. Apoplastic washing fluid (AWF) was collected by vacuum-infiltration and centrifugation of leaves. Means of 14 replicates each day are significantly different at $p<0.05$ (Tukey) as indicated by different letters. Coefficients of determination of regression analysis are significant as ***, **, * for $p<0.001$, 0.01 and 0.05.

After 2 days of Mn treatment, first visible Mn toxicity symptoms (brown spots) were detectable (Fig. 2). The development of Mn toxicity symptoms was significantly correlated with the Mn tissue content (Fig. 2).

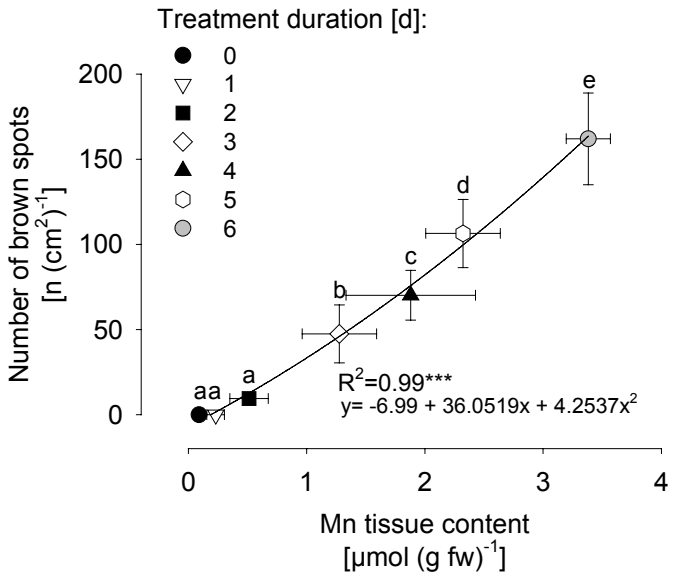

Figure 2 Relationship between the Mn tissue content and the number of brown spots on the trifoliate leaves of cowpea. Four leaf discs per leaf were incubated in ethanol to remove chlorophyll. The number of brown spots was then determined by direct counting. Means of 14 replicates each day are significantly different at $p<0.05$ (Tukey) as indicated by different letters. Coefficients of determination of regression analysis are significant as ***, **, * for $p<0.001$, 0.01 and 0.05.

An equally sensitive indicator of Mn toxicity appears to be the induction of callose synthesis in the leaves (Fig. 3). After 2 days of Mn treatment, callose content started to increase and was significantly higher after 3 days.

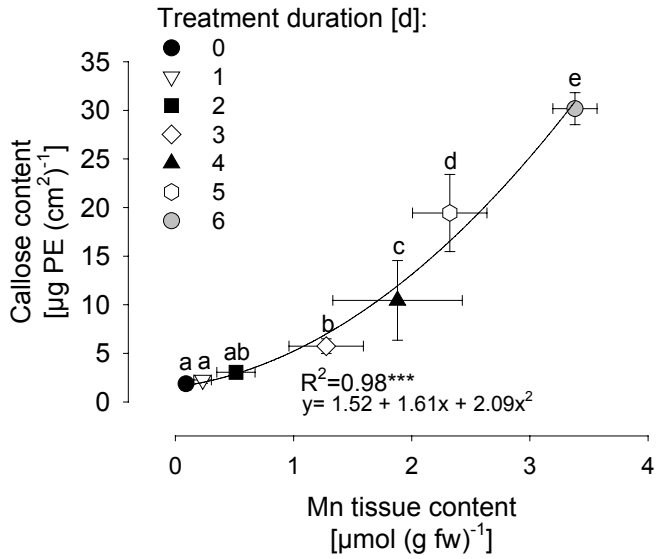

Figure 3 Relationship between the Mn and callose contents of leaves. Means of 14 replicates each day are significantly different at $p<0.05$ (Tukey) as indicated by different letters. Leaf discs, previously used for counting brown spots, were homogenized in NaOH. The extracted callose was detected by anilin blue staining. Coefficients of determination of regression analysis are significant as ***, **, * for $p<0.001$, 0.01 and 0.05.

The activities of both guaiacol- and NADH-PODs (Fig. 4, A and B) in the leaf AWF increased after 2 days of exposure to elevated Mn supply. This was accompanied by the release of proteins into the apoplast (Fig. 4C). In summary, formation of brown spots, activity of enzymes, the protein concentration, and the callose induction were significantly correlated with the Mn tissue contents (Figs. 2-4).

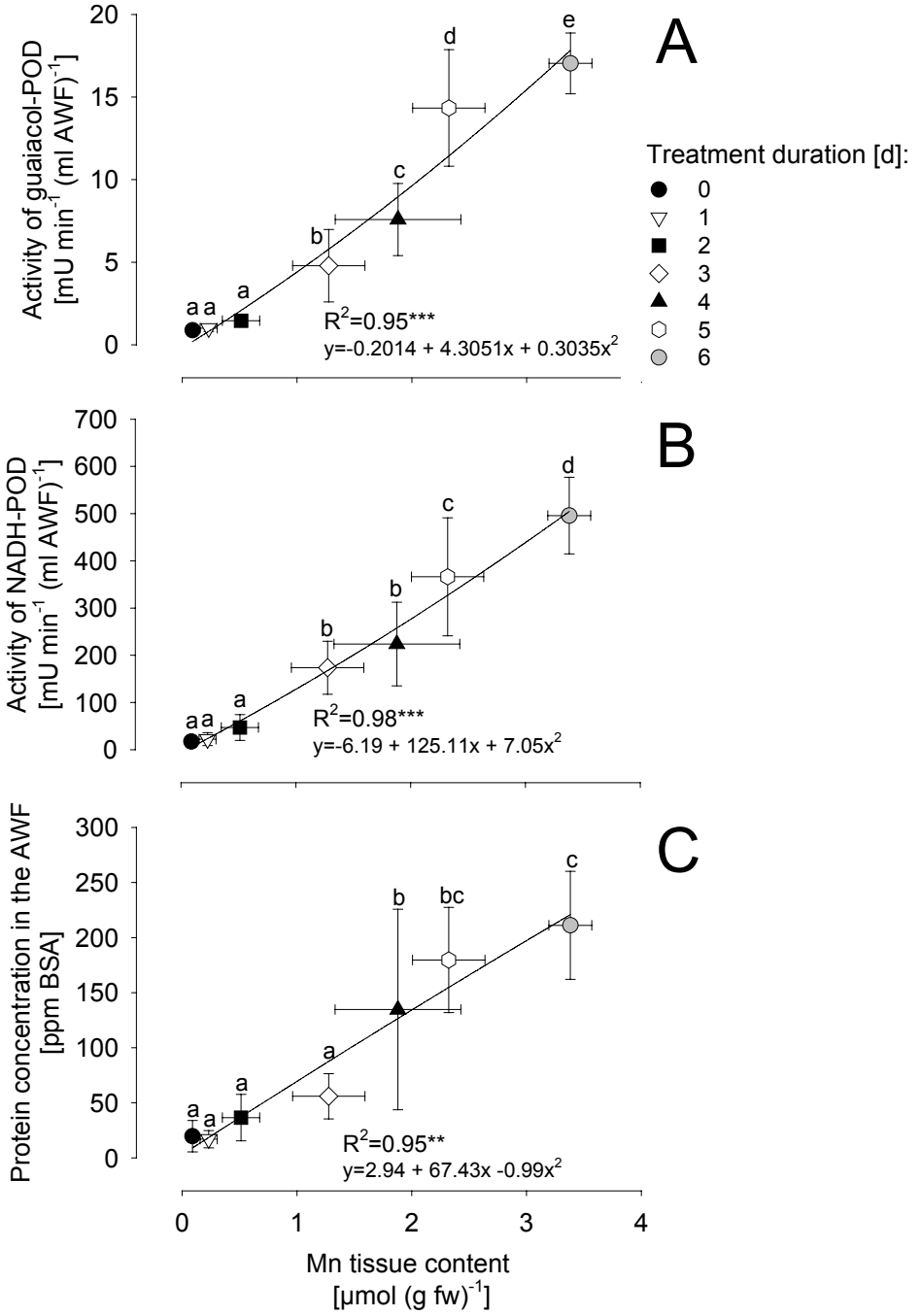

Figure 4 Relationships between the Mn tissue content and (A) the activity of guaiacol-peroxidase, (B) the activity of NADH-*per*oxidase and (C) the concentration of total protein in the AWF of leaves. Guaiacol-POD activity was measured in the presence of 20 mM guaiacol and 0.03% (w/w) H_2O_2 (pH 6). NADH-*per*oxidase was measured in the presence of 0.6 mM NADH, 1.6 mM p-coumaric acid and 16 mM $MnCl_2$ (pH 5). Protein concentrations were measured according to Bradford (1976). Means of 14 replicates each day are significantly different at $p<0.05$ (Tukey) as indicated by different letters. Coefficients of determination of regression analysis are significant as ***, **, * for $p<0.001$, 0.01 and 0.05.

Analysis of water-soluble proteins from the leaf apoplast by 2D Blue-Native (BN)/SDS-PAGE and LC-MS/MS

To systematically monitor the induction of apoplastic proteins upon Mn treatment, AWF was analysed by BN-PAGE. For this procedure, Coomassie dyes are used before to electrophoresis to introduce negative charges into proteins and protein complexes without denaturing them. BN-PAGE allows proteins to be efficiently resolved under native conditions. Subsequently, proteins can be visualized by Coomassie Blue staining and PODs by in-gel activity measurements using guaiacol as a substrate, which leads to the formation of a brown-coloured oxidation product (Fig. 5). Mn treatment causes an induction of several apoplastic PODs in the 30-kD range (Fig. 5A). Before treatment (d=0), POD activity was expressed by two to three bands in the gel. These bands became stronger upon prolonged Mn treatment. Furthermore, new bands became visible after 2 days of Mn treatment (Fig. 5A). The staining of the gel with Coomassie Blue showed additional proteins, which also were induced by Mn treatment (Fig. 5B).

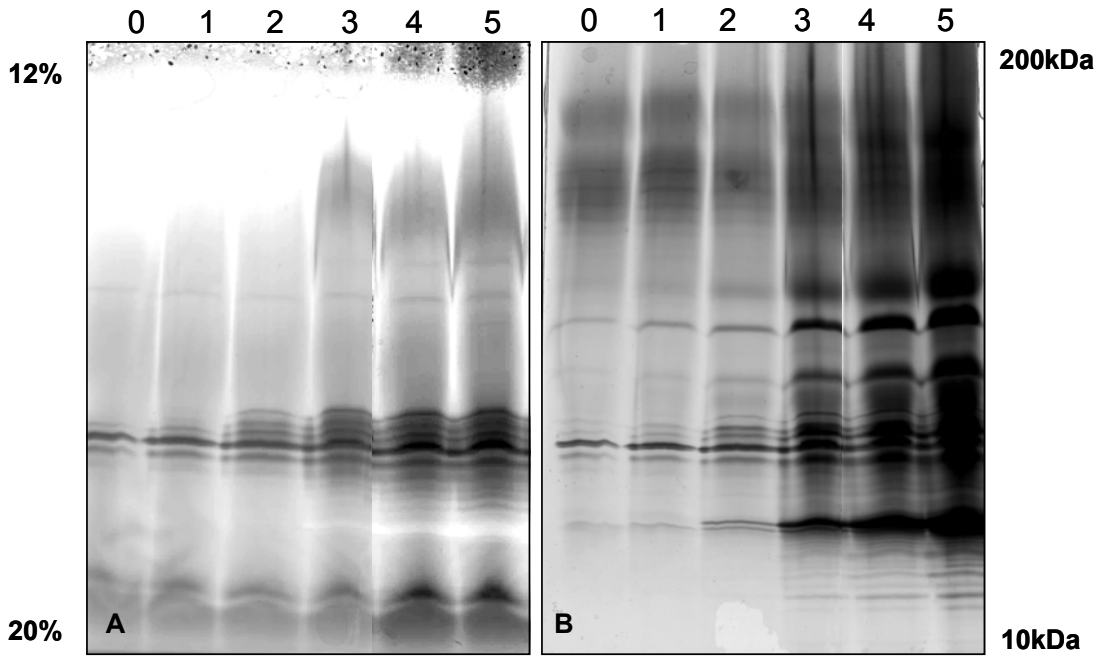

Figure 5 1D resolution of water-soluble proteins from the leaf apoplast of cowpea. Separation was carried out by Blue-native (BN)-PAGE using a 12% to 20% gradient gel. Peroxidases were detected by staining with 20 mM Guaiacol + 0.01% (w/w) H_2O_2 (A) and total protein with colloidal Coomassie-blue (B). The numbers above the gels indicate duration of the Mn treatment (in days), numbers on the right indicate molecular masses of proteins.

Because the Mn toxicity-induced PODs have very similar molecular masses, BN gel electrophoresis was combined with SDS-PAGE to increase resolution capacity (Fig. 6). Most protein bands of the native gel dimension were separated into at least two protein spots in the second gel dimension. Several protein spots became specifically visible upon Mn treatment (Fig. 6B).

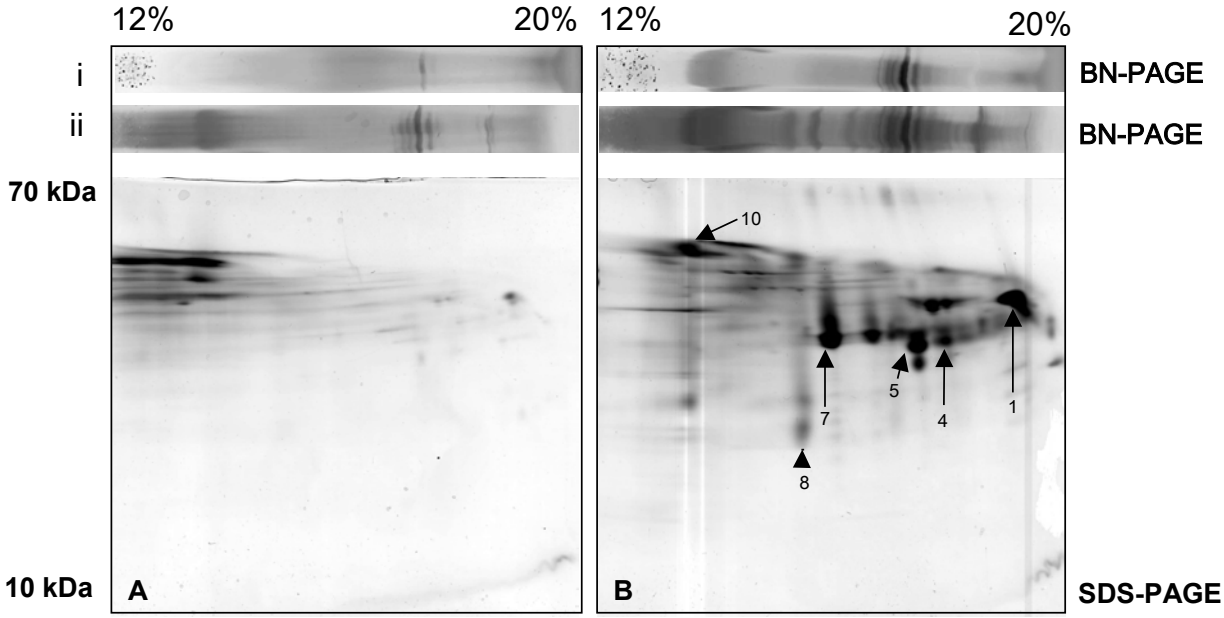

Figure 6 2D resolution of water-soluble proteins from the leaf apoplast of cowpea by Blue-native/SDS-page. Peroxidases were detected by staining with 20 mM guaiacol + H_2O_2 (i) and total proteins with colloidal Coomassie-blue (ii and 2nd dimension). Plants precultured for 13 days were treated with 50 µM Mn for 5 days (B), while control plants received 0.2 µM Mn continuously (A). Numbers on the top of the gels indicate the acrylamide concentration, and the numbers on the right indicate molecular masses of proteins. Marked spots were identified by nano LC-MS/MS.

Representative spots of the constitutive POD and Mn-induced proteins were cut out of the gel and analysed by LC-MS/MS (Table 1). The sequence of one peptide of protein no 4 exhibits significant sequence identity to previously characterized PODs from other plant species (Table 1). Additional bands identified as POD by guaiacol staining showed sequence similarities to thaumatin-like proteins (spot 5). Further Mn-induced proteins were identified as chitinases (spot 1 and 7), glucanases (spot 1), fascilin-like arabinogalactan-protein (spot 7), hevein-like and wound-induced proteins (spot 8), and pathogenesis-related (PR) proteins class 1 (spot 10). Most of the proteins show similar molecular masses in the range of 20-30 kD, which caused overlappings of proteins on 1D and 2D gels.

Table 1 . Identified proteins of the apoplast of cowpea (*Vigna unguiculata*) after separation by BN-/SDS-PAGE

No[a]	Ion[b]	Sequence[c]		Identified protein[d]	Accession No.	Matching sequence	pI[e]	MW[e]	Reference
1	992.38	QEVVDLYK	*	1,3-beta-glucanase [Glycine max] (Soybean)	T05957	QEVVDLYK	4.27	26074.11	
	1074	HFGLFNPDK		1,3-beta-glucosidase [Hoderum vulgare] (Barley)	AAA32960	HFGLFNPDK	8.46	32583.82	Xu,P. et al., 1992
	1266.36	VNYYTEYCR		Chitinase class 4 [Vigna unguiculata] (Cowpea)	S57476	VNYYTEYCR	4.25	26876.36	
4	762.32	MGASLLR		Peroxidase [Triticum aestivum] (Wheat)	AAM76682	MGASLLR	8.89	32425.66	
				Peroxidase POC1 [Oryza sativa] (Rice)	AAF65464	MGASLLR	6.40	32461.60	
5	978	GSDGSVLGCK	*	Thaumatin-like protein 1 precursor [Pyrus pyrifolia] (Sand pear)	O80327	GSDGSVIGCK	5.07	25308.31	Sassa,H. and Hirano,H. 1998
	1693	SACLAMGDDQYCCK	*	Thaumatin-like protein 1 precursor [Pyrus pyrifolia] (Sand pear)	O80327	SACLALNQPQYCC	5.07	25308.31	Sassa,H. and Hirano,H. 1998
	1069.42	TGCNFDGDGK		Thaumatin-like protein PR-5a [Cicer arietinum] (Chickpea)	CAA09229	TGCNFDGSG	5.51	18732.00	
				Pathogenesis-related protein 5 precursor [Arabidopsis thaliana]	JQ1695	TGCNFDASGNG	4.75	25252.31	Uknes, S. et al., 1992
	898	LPSPWSGR		Thaumatine-like protein [Malus x domestica] (Apple tree)	AAM12886	PSPWSGR	4.60	22107.61	Venisse, J.S. et al., 2002
7	1095	YGGVMLWDR	*	Chitinase (EC 3.2.1.14) class III, acidic [Glycine max] (Soybean)	T05187	YGGVMLWDR	4.96	37110.79	Yeboah, N.A. et al., 1998
	1909	CNPSLNCNVFSDLQK	*	Chitinase (EC 3.2.1.14) class III, acidic [Vigna unguiculata] (Cowpea)	S57468	CNPSINNCNVFSD	4.81	26326.12	
	966	VGFGSAASGSK		Endosperm-specific protein-like protein [Arabidopsis thaliana].	AAM66074	VGFGSAASGSK	5.43	43060.81	Haas, B.J. et al., 2002
				Fasciclin-like arabinogalactan-protein [Arabidopsis thaliana]	NP_566043	VGFGSAASGSK	5.43	43074.83	
	1434	QQFPLAVXXXDK		Fasciclin-like arabinogalactan-protein (FLA6) [Arabidopsis thaliana]	NP_565475	QQFPLAV	5.51	26506.10	
8	1454	AVSAYCSTYDADK		Hevein-like protein precursor [Arabidopsis thaliana]	P43082	AVSAYCSTWDADK	7.89	22936.63	Potter,S. et al., 1993
				Wound-induced protein (clone TAB7) [Lycopersicon esculentum] (Tomato)	T07729	AVSAYCSTWDANK	8.38	21607.17	Harris,N. et al., 1997
			*	Putative pathogen-induced protein [Lycopersicon esculentum] (Tomato)	CAC81819	AVSAYCSTWDANK	6.62	14960.57	
10	1636	YDYGSNTCVGGECR		PR-1 protein [Vitis vinifera] (Wine grape)	CAA05868	YDYNSNSCVGGEC	6.05	11078.28	
	1013	LWVDEKP		Pathogenesis-related protein 1-1a [Cucumis sativus] (Cucumber)	AAL84768	LWVDEKP	6.92	15655.49	
	1453	YGENLAGSSGDLSG	*	Pathogenesis-related protein 1 [Betula pendula] (European white birch)	AAF62171	YGENLAASSGDLSG	5.99	10819.80	

a)The numbers correspond to the numbers given in Figure 6
b)Ion precursor (mass/charge unit)
c)Amino acid sequence as determined by ESI-MS/MS.
Due to similar molecular properties, leucine and isoleucine cannot be distinguished. L represents both.
d)Proteins were identified by http://www.ncbi.nlm.nih.gov/BLAST/ . Stars indicate correspondences to Table 2.
e)Isoelectric point and molecular mass were calculated by http://www.expasy.org/

To further improve resolution of proteins from the AWF, a second two-dimensional gel system was employed, which is based on isoelectric focussing (IEF) for the first gel dimension and SDS-PAGE for the second (Fig. 7).

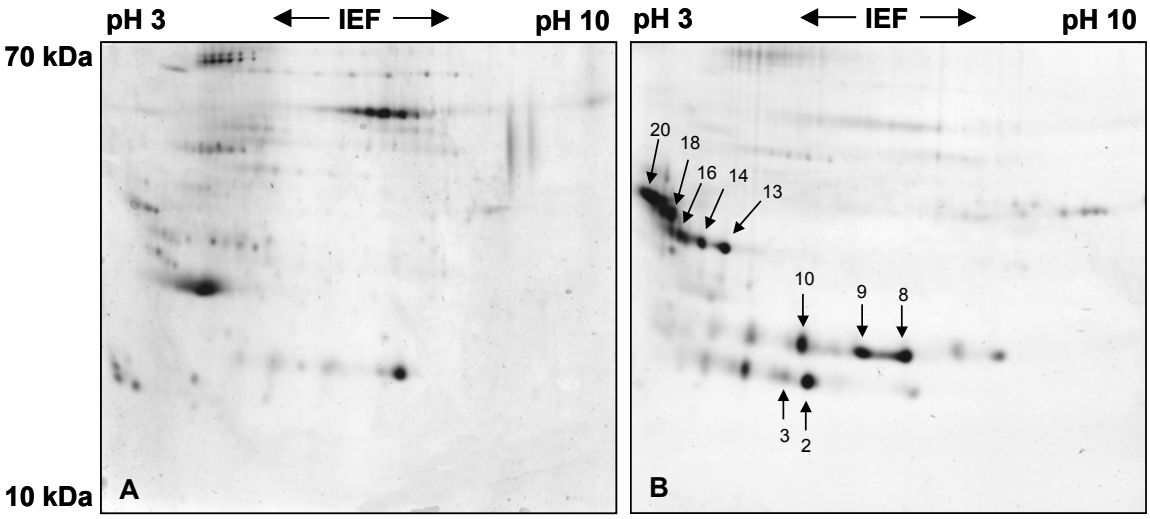

Figure 7 2D resolution of water-soluble proteins from the leaf apoplast of cowpea by IEF/SDS-PAGE. Plants precultured for 13 days were treated with 50 µM Mn for 5 days (B), while control plants received 0.2 µM Mn continuously (A). Numbers on the top of the gels indicate the pH gradient, and the numbers on the left indicate molecular masses of proteins. Marked spots were identified by nano LC-MS/MS.

Table 2 . Identified proteins of the apoplast of cowpea (*Vigna unguiculata*) separated by IEF/SDS-PAGE

No[a]	Ion[b]	Sequence[c]		Identified protein[d]	Accession No.	Matching sequence	pI[e]	MW[e]	Reference
2	1449.48	SAYCSTYDA	*	Putative pathogen-induced protein [Lycopersicon esculentum] (Tomato)	CAC81819	SAYCSTWDA	6.62	14960.57	
				Wound-induced protein 2 percursor [Solanum tuberosum] (Potato)	S04927	SAYCSTWDA	7.37	22498.23	Stanford, A. et al., 1989
				Hevein-like protein precursor [Arabidopsis thaliana]	P43082	SAYCSTWDA	7.89	22936.63	Potter, S. et al., 1993
3	1449.57	GLSAYCRQ		?					
8	1652.52	NRDYGSNTCVG		Pathogenesis related protein [Hordeum vulgare] (Barley)	CAA52893	DYGSNTCAG	8.19	17439.62	Bryngelson, T. et al., 1994
	2794.21	VAA(M/F)A(Q/K)NYANQR		Pathogenesis related protein [Nicotiana tabacum] (Tobacco)	S22531	AAFAQNYANQR	7.62	20120.38	Eyal, A. et al., 1992
	1714.56	ESPEADYLLNEHNAAR		Pathogenesis related protein [Nicotiana tabacum] (Tobacco)	S22531	SPQ-DYL-NPHNAAR	7.62	20120.38	Eyal, A. et al., 1992
9	1637.52	YGENLAGSSGDLSGK	*	Pathogenesis related protein [Betula pendula] (Birch)	AAF62171	YGENLAASSGDLSG	5.99	10819.8	
10	1636.52	QNYGSNTCVGWCR		Pathogenesis related protein precursor [Hordeum vulgare] (Barley)	S52627	NYGSNTC	9.08	17678.86	Mouradov, A. et al.,1994
13	1722.72	SSWNQWTS	*	Chitinase class III, acidic [Vigna unguiculata] (Cowpea)	S57468	SSWNQWTS	4.81	26326.12	
		YGGVMLDSDR	*	Chitinase class III, acidic [Glycine max] (Soybean)	T05187	YGGVML-WDR	4.96	37110.79	Yeboah, N.A. et al., 1998
14	1721.74	(LS)LSSWNQWTSSQAK		Chitinase [Vigna angularis] (Adzuki bean)	P29024	SSWNQWTSSQAK	4.9	31701.55	Ishige, F. et al., 1993
16	1721.74	YGGV(F/M)LWDR		Chitinase class III, acidic [Glycine max] (Soybean)	T05187	YGGVMLWDR	4.96	37110.79	Yeboah, N.A. et al., 1998
	945.46	AL(N/D)GLSSQR		Chitinase class III [Sphenostylis stenocarpa] (African yam-bean)	AAD27874	ALNDLSSQR	4.08	31392.93	Colucci, G. et al., 1999
				Putative peroxidase [Oryza sativa] (Rice)	AAL34128	LDGLSS	4.65	33956.4	
	1222.46	EGTLAETCNTK		Chitinase class III [Sphenostylis stenocarpa] (African yam-bean)	AAD27874	EGTLADTCNT	4.08	31392.93	Colucci, G. et al., 1999
18	1060.57	HFGLFTPDK		1,3-beta-glucanase [Zea mays] (Maize)	T02088	HFGLFNPDK	4.56	34893.92	Wu, S. et al., 1994
	1559.79	TQLNAACPWLKMK		Peroxidase, cationic [Stylosanthes humilis] (Townsville stylo)	AAB67737	TQLEAACP	7.06	33781.18	Stines, A.P. et al., 1996
	978.45	(GS)DGSVLGCK	*	Thaumatin-like protein 1 precursor. [Pyrus pyrifolia] (Sand pear)	O80327	DGSVIGCK	5.07	25308.31	Sassa,H. and Hirano,H. 1998
20	992.54	KEVVDLYK	*	1,3-beta-glucanase [Glycine max] (Soybean)	T05957	EVVDLYK	4.27	26074.11	
	974.57	AAPVVDLYK		Glucan endo-1,3-beta-d-glucosidase [Cicer arietinum] (Chickpea)	CAA10167	VVDLYK	5.49	35595.06	
	1385.77	QRGLFNPDKSPK		Glucan endo-1,3-beta-d-glucosidase [Cicer arietinum] (Chickpea)	CAA10167	GLFNPDKSPK	5.49	35595.06	

a)The numbers correspond to the numbers given in Figure 7
b)Ion precursor (mass/charge unit)
c)Amino acid sequence as determined by ESI-MS/MS.
Due to similar molecular properties, leucine and isoleucine cannot be distinguished. L represents both.
d)Proteins were identified by http://www.ncbi.nlm.nih.gov/BLAST/ . Stars indicate correspondences to Table 1.
e)Isoelectric point and molecular mass were calculated by http://www.expasy.org/

Analysis of water-soluble proteins from the leaf apoplast by IEF/ SDS-PAGE and LC-MS/MS

In agreement with the results obtained by 2D BN-PAGE, 2D IEF/SDS-PAGE allowed visualization of several proteins, which are specifically induced upon Mn treatment, including a group of proteins in the 25-30 kD range (proteins nos. 2, 3, 8, 9, and 10 on Fig. 7B). These proteins are clustered at pH 6-9. Another group of proteins can be found in the 40 kD range at acidic pH (proteins nos. 13, 14, 16, 18, and 20). Analysis of selected protein spots by nano LC-MS/MS allowed determination of peptide sequences (Table 2). Based on sequence similarity to characterize proteins from other organisms, the analysed proteins represent PR proteins (spot 2, 8, 9, and 10), chitinases (spot 13, 14, and 16), 1,3-beta-glucanases (spot 18 and 20) and PODs (spot 16 and 18).

DISCUSSION

PODs in the leaf apoplast

The leaf apoplast is a compartment of storage and physiological reactions like intercellular signalling, defense against biotic and abiotic stresses, and transport of water and nutrients (Sakurai, 1998; Sattelmacher, 2001). The extracellular space of plants contains acidic (anionic) and basic (cationic) POD-isoenzymes present both as cell wall-bound and soluble enzymes with differential affinities to substrates (Campa, 1991; Kärkönen et al., 2002). The acidic PODs show a high affinity to lignin precursors and H_2O_2 and were considered to be important for the normal functioning of the cell wall (Mäder et al., 1980; Imberty et al., 1985; Ros Barcelo et al., 1987). The expression of PODs is tissue-specific and regulated by development (Mohan et al., 1993; Klotz et al., 1998). In tobacco (*Nicotiana tabacum*), acidic PODs were strongly expressed in trichomes and the epidermis (Klotz et al., 1998) and not expressed in tissues or regions undergoing growth, probably due to the inhibitory effect of PODs on growth and elongation (MacAdam et al., 1992a+b; de Souza and MacAdam, 1998). Acidic PODs in the apoplast of tobacco were proposed to be involved in the first level of defence reactions against several stresses (Klotz et al., 1998). This was indicated by the induction of PODs and POD promoters by wounding, pathogen attack (Lagrimi and Rothstein, 1987; Mohan et al., 1993), and Cu treatment (Cuypers et al., 2002).

PODs were also proposed to be able to produce H_2O_2 necessary for lignification (Mäder and Amberg-Fisher, 1982). In general, basic PODs show a high affinity to NADH with

subsequent formation of H_2O_2. The NADH-oxidation by POD is strongly stimulated in the presence of Mn and monophenolic compounds (Halliwell, 1978). Therefore, NADH-POD activities were mostly measured in the presence of Mn and phenols (Otter and Polle, 1997; Chi and Kao, 2001). The H_2O_2 production and consumption is probably catalysed independently by different PODs as suggested by Mäder et al. (1980). A two-step control of PODs in the apoplast was described by Gaspar et al. (1985). The rapid increase of activity of basic PODs was ascribed to demasking effects of available PODs in the apoplast or by a rapid secretion of basic PODs into the apoplast under the control of Ca^{2+} (Castillo et al., 1984). Basic PODs responded to several stimuli within seconds. The secretion of acidic PODs was assumed to occur at a later stage, stimulated by ethylene and Ca^{2+}. This was proposed to be linked to the release of phenolic monomers with subsequent cross-link reactions catalysed by acidic PODs (Castillo, 1986).

Additionally, PODs were also proposed to act as polyfunctional enzymes operating simultaneously as oxidase and POD (Pedreño et al., 1995), dependent from pH and availability of reducing compounds, e.g. NADH and IAA (Penel, 1995).

Relationship between Mn toxicity and POD in the leaf apoplast of cowpea

In cowpea, the synchronous increase in POD and the formation of brown spots suggest a close relationship between the expression of Mn toxicity symptoms and the activity of water-soluble POD extracted from the apoplast (Figs. 2 and 4). The Mn-induced increase of POD activity was also reflected by the induced presence of several proteins on BN-gels (Fig. 5). Among these, one protein of approximately 32 kD was identified as POD on the basis of sequence similarity to known PODs from other organisms (no. 4 in Fig. 6 / Table 1). PODs, which specifically were induced upon Mn treatment, could also be identified by 2D IEF/SDS-PAGE in the acidic pH range (Fig. 7). The estimated pI values indicate that these POD isoenzymes belong to the group of acidic PODs. These pI values differ in some cases from the pI values reported in the literature. This discrepancy might be due to the broad variance of POD isoenzymes in plants. Even within plant species, POD isoenzymes may differ by more than 50% in peptide sequence (Welinder, 1992). Therefore, identification of PODs on the basis of sequence similarities is difficult. The browning of the leaf tissue in cowpea in response to toxic Mn supply might be due to the oxidation of phenolic compounds especially by H_2O_2-consuming acidic PODs, which have a high affinity to monophenols in the leaf apoplast. The Mn toxicity symptoms occur in organs

with reportedly high POD expression, e.g. epidermis and trichomes of older plant organs. The spatial distribution of POD expression could be the cause for the formation of Mn toxicity symptoms especially in the epidermis of cowpea. This relationship is confirmed by observations in sunflower (*Helianthus annuus*; Blamey et al., 1986), Arabidopsis, tobacco, and rape seed (W.J. Horst, P. Maier, and M.M. Fecht-Christoffers, unpublished data). Mn toxicity in these species leads to brown depositions in and at the base of trichomes. Additionally, leave age plays a major role for the expression of Mn toxicity symptoms. In cowpea, older leaves are significantly more sensitive than young leaves at elevated Mn tissue contents (Horst, 1988). Also in tobacco, Mn-induced formation of brown spots was observed exclusively on older leaves (W.J. Horst, P. Maier, and M.M. Fecht-Christoffers, unpublished data).

The NADH-oxidation rate showed a similar reaction pattern as the "guaiacol-PODs", indicating that PODs in the apoplast of cowpea were able to react with guaiacol and NADH as well (Fig. 4). After the first day of treatment, a slight increase of NADH-POD was detectable without a marked change in POD-isoenzyme composition. This was accompanied by a slight increase in callose formation. The physiological role of callose formation in response to toxic Mn levels in the tissue is unknown, but its detection serves as an additional sensitive parameter for Mn-induced injury of the leaf tissue (Wissemeier and Horst, 1987). A Mn-enhanced release of H_2O_2 from cowpea-leaf segments (Horst et al., 1999) and from soybean cells grown in suspension culture (W.J. Horst, P. Maier, and M.M. Fecht-Christoffers, unpublished data) was observed, indicating that Mn stimulates the H_2O_2-production in the leaf apoplast. Assuming 5 to 10-fold higher apoplastic Mn concentrations *in vivo* than in the AWF (Lohaus et al., 2001), a direct enhancement of the NADH-POD activity by Mn (10 to 160 μM) can not be excluded leading to H_2O_2 production and subsequent stimulation of H_2O_2-consuming PODs. It remains unclear whether H_2O_2 production or H_2O_2 consumption is preferred by apoplastic water-soluble PODs *in vivo*. Since a high spatial variability of apoplastic pH in leaves has been demonstrated (Hoffmann and Kosegarten, 1995; Sattelmacher et al., 1998; Mühling and Läuchli, 2000), a relationship with the typically uneven distribution of Mn toxicity symptoms in the leaves (Horst and Marschner, 1978; Horst, 1980) could be assumed.

Other proteins in the apoplast that are induced by Mn stress

The release of PODs in the apoplast was accompanied by the secretion of proteins, which show similarities to wound-induced proteins and PR enzymes, e.g. glucanase, chitinase and thaumatin-like proteins and PR proteins class I. Furthermore, the gels in Figures 6 and 7 still exhibit regions with high spot density, indicating that further proteins might exist that overlap in the 2D gels. This fact is also reflected by the identification of peptides from single protein spots, which exhibit sequence identity to different proteins from databases. Despite this variability, the partial agreement between the data in Tables 1 and 2 demonstrate their reproducibility. PR-denominated proteins in plant tissues are induced by pathogens, and their expression often was confirmed independently for more than one plant-pathogen combination. Homologous proteins not induced by pathogens are denominated PR-like proteins (van Loon and van Strien, 1999). The Mn toxicity-induced proteins released into the AWF of cowpea leaves show similarities to PR-like proteins induced by heavy metals (Didierjean et al., 1996), during plant development (Malehorn et al., 1993) and senescence (Hanfrey et al., 1996), by wounding (Standford et al., 1989; Ishige et al., 1993; Harris et al., 1997), and by ripening of fruits (Fils-Lycaon et al., 1996; Ruperti et al, 2002). Their induction was related to the presence of plant signalling-molecules, e.g. ethylene (Watanabe et al., 1999), abscisic acid (Akiyama and Pillai, 2001) and salicylic acid (Uknes et al., 1992; Busam et al. 1997). In general, PR-like proteins were reported to be induced by several abiotic stresses, e.g. ozone (Schraudner et al., 1992; Yalpani et al., 1994), UV irradiation (Didierjean et al., 1996; Rakwal et al., 1999), heat stress (Margis-Pinheiro et al., 1994), wounding (Zhang and Punja, 1994; Ruperti et al., 2002), heavy metals (Edreva 1990; Jacobsen et al., 1992; Rakwal et al., 1999), and during salt (Esaka et al., 1994) and cold-adaptation (Antikainen et al., 1996; Hiilovaara-Teijo et al., 1999). Mn-induced expression of PR-like proteins was observed in tobacco (Edreva, 1990) and sunflower leaves (Jung et al., 1995), whereas several PR-proteins were also induced by a physiological, non-pathogenic disorder (Edreva, 1990), mercury and UV light (Jung et al., 1995). These findings led to the conclusion that the Mn-induced secretion of the PR-like proteins is part of a general stress response of plants. This unspecific induction of PR-like proteins was also observed in maize plants treated with mercury chloride and gave evidence of the presence of multiple pathways of gene regulation in response to abiotic stresses (Didierjean et al., 1996). The induction of PR-like proteins during development and cold adaptation was proposed to enhance resistance against other abiotic

and biotic stresses (Antikainen et al., 1996; Hiilovaara-Teijo et al., 1999; Ruperti et al., 2002). In cowpea, a potential beneficial effect of induced PR-like proteins is unknown and to this date not considered. The development of symptoms accompanied by the release of proteins into the apoplast represents a sensitive response towards increased Mn supply leading to reduced plant growth and yield production.

CONCLUSIONS

In this work, physiological and proteomic changes were studied at different stages of Mn toxicity to get a better understanding of the role of Mn-induced processes in the leaf apoplast in the expression of Mn toxicity. The appearance of Mn toxicity symptoms was preceded by a steep increase of the Mn concentration of the AWF indicating a particular role of free apoplastic Mn in the expression of Mn toxicity despite of its low contribution to the total Mn content of the leaf. Mn excess almost simultaneously induced the formation of brown spots and callose, the activation of guaiacol- and NADH-PODs, and the release of proteins into the apoplast. The analysis of the proteome of the leaf apoplast has placed previous results and speculations about the physiology of Mn toxicity (Horst et al, 1999; Fecht-Christoffers et al., 2003) on a more solid basis. They confirm the particular role of PODs in the expression of Mn toxicity mediating H_2O_2 production/consumption and the oxidation of phenols in the leaf apoplast. However, on the base of these observations, conclusions about the chronology of Mn-induced physiological changes are difficult to draw. Particularly, the enhanced release into the apoplast of PR-like proteins appears to be a late response to excess Mn. A more detailed kinetic study with emphasis on very early stages on Mn toxicity and a comparison of Mn-sensitive and Mn-tolerant leaves (genotype, Si nutrition, leaf age) are required.

CHAPTER 3:

THE ROLE OF H_2O_2-PRODUCING AND H_2O_2-CONSUMING PEROXIDASES IN THE LEAF APOPLAST OF *VIGNA UNGUICULATA* L. IN MANGANESE TOXICITY

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(to be submitted)

ABSTRACT

The apoplast is considered the leaf compartment decisive for the development and avoidance of Mn toxicity. Particularly apoplastic peroxidases (PODs) were proposed to be key enzymes in Mn toxicity-induced processes. So far, the emphasis was laid on H_2O_2-consuming PODs. However, PODs possess polyfunctionality: they act as H_2O_2-producing and H_2O_2-consuming enzymes in the apoplast. The presented work focuses on the characterization of H_2O_2-producing (NADH-*per*oxidase) and H_2O_2-consuming peroxidase (guaiacol-POD) in the apoplastic washing fluid (AWF) of leaves of cowpea cultivars differing in Mn tolerance and the factors affecting the resulting H_2O_2 formation.

Proteins purified from leaf AWF showed a strong increase of H_2O_2-consuming guaiacol-POD and of H_2O_2-producing NADH-*per*oxidase activities at elevated AWF Mn concentrations only in the Mn-sensitive cultivar TVu 91. This is also reflected by 2D resultions of AWF proteins showing that the AWF protein pattern of the Mn-tolerant cultivar TVu 1987 remained nearly unaffected by high Mn treatment whereas the Mn-sensitive cv TVu 91 expressed more and additional proteins. The NADH-*per*oxidase activities from control and Mn-treated plants correlated positively linearly with the *in vitro* release of H_2O_2, confirming a role of the NADH-*per*oxidase system in H_2O_2 formation. A linear relationship between both parameters was also observed in the leaf AWF of the Mn-tolerant cultivar TVu 1987 but at a lower level. In both cultivars, NADH-*per*oxidase activity and H_2O_2 formation of AWF proteins *in vitro* were significantly stimulated by Mn^{2+} and p-coumaric acid. To investigate the effect of naturally occurring co-factors in the AWF on the NADH-*per*oxidase activities, proteins from the AWF were separated from non-protein compounds. The protein fractions and the non-protein fractions from controls and Mn-treated plants form both cultivars were crosswise combined and the NADH-*per*oxidase activity was measured. The nature of the non-protein fraction had a significant effect on NADH-*per*oxidase activity. The initial phase was significantly delayed in the presence of the non-protein fraction from the Mn-tolerant cv TVu 1987, whereas the non-protein fraction from the Mn-sensitive cv TVu 91 shorted lag phase. Since phenols have been shown to affect NADH-*per*oxidase, phenolic compounds in the leaf AWF were studied. The density of brown spots was closely positively related to the total phenol concentration in the AWF of the Mn-sensitive cv TVu 91. However, the HPLC

chromatogram of acid hydrolysates of the leaf AWF phenols of the Mn-tolerant cv TVu 1987 indicate the presence of phenols not observed in the Mn-sensitive cv TVu 91.
We conclude from our results, that PODs in the leaf apoplast of cowpea are capable to produce and to consume H_2O_2 in the apoplast. For the development and avoidance of Mn toxicity, the H_2O_2-producing NADH oxidase cycle and its modulation by stimulating or inhibiting phenolic compounds in the leaf apoplast seem to play a decisive role.

INTRODUCTION

Manganese (Mn) excess represents an important factor limiting growth and crop yield particularly on acid and insufficiently drained soils with low redox potential (Foy 1984, Schlichting and Sparrow, 1988). The oxidation of Mn^{II} and phenolic compounds catalysed by peroxidase (class III; EC 1.11.1.7) in the leaf apoplast is considered as the key reaction leading to Mn toxicity symptoms and finally leaf injury in cowpea (*Vigna unguiculata*) (Horst 1988). This assumption is based mainly on two observations: (i) first Mn toxicity symptoms (brown spots) occur in the cell wall and consist of oxidized Mn and oxidized phenolic compounds (Horiguchi, 1987; Wissemeier and Horst, 1992), (ii) Kenten and Mann (1950, 1956) observed a close relationship between peroxidase-catalysed phenol and Mn oxidation. The oxidation of Mn^{II} and phenols is mediated by the formation of highly reactive Mn^{III} and phenoxyradicals, which are considered as phytotoxic agents (Horst, 1988, Horst et al., 1999). Particular the simultaneous enhancement of POD activity and expression of Mn toxicity symptoms (brown spots) (Fecht-Christoffers et al., 2003a,b) suggest a close relationship between PODs and the oxidation of Mn and phenolic compounds. However, the activation of PODs by Mn excess in cotton was considered a secondary effect (Sirkar and Amin, 1974), and also from our own work in cowpea it cannot be decided yet, whether the enhanced apoplastic POD activity is the reason or the consequence of Mn-induced leaf injury.
Peroxidases are widely distributed in plant tissues with a broad variance in functionality, associated with an apparent lack in substrate specificity (Campa, 1991). In the plant apoplast, they were attributed to act as H_2O_2-consuming and phenol-oxidizing enzymes, thus leading to secondary cell wall and lignin formation (Polle et al., 1994; Sato et al., 1995; Christensen et al., 1998; Kärkönen et al., 2002). Peroxidases using NADH as electron donor were also termed `NADH oxidases´ and have been suggested to play an important role in the formation of H_2O_2 needed for lignification (Elstner and Heupel, 1976; Gross et al., 1977; Halliwell, 1978; Mäder et al., 1980; Mäder and Amberg-Fisher, 1982;

Bolwell et al., 1995). Manganese and phenolic compounds presumably control the activity of the NADH-*per*oxidase (Elstner and Heupel, 1976; Gross et al., 1977; Halliwell, 1978; Mäder and Füssl, 1982; Stich and Ebermann, 1984; Pedreño et al., 1987). Proteins purified from the apoplastic washing fluid (AWF) of leaves from the Mn-sensitive cowpea cultivar TVu 91 showed NADH-*per*oxidase activity, which was strongly enhanced at high leaf Mn-concentrations (Fecht-Christoffers et al., 2003b). The stimulation of this H_2O_2-producing NADH-*per*oxidase in the leaf apoplast of Mn-treated plants might by the cause for the elevated H_2O_2 formation of washed intact leaf segments from cowpea showing Mn toxicity symptoms (Horst et al., 1999).

Plant species and cultivars within species show considerable differences in Mn tolerance. Several mechanisms of Mn tolerance have been proposed: (i) sequestration of Mn in the apoplast thus preventing Mn oxidation in the apoplast (Maier, 1997; Horst and Maier, 1999), (ii) transport of Mn into the vacuoles (Hirschi et al., 2000; Schaaf et al., 2002) and sequestration by organic acids (Maier, 1997; Horst and Maier, 1999), (iii) maintaining low cytosolic Mn concentration by enhanced transport of Mn into the ER (Wu et al., 2002), and (iv) scavenging the Mn-induced enhanced formation of reactive oxygen species (ROS), phenoxy radicals and Mn^{III} by an efficient ascorbic acid turnover in the apoplast and cytoplasm (Fecht-Christoffers et al., 2003a). But none of the factors (i), (ii) and (iv) can satisfactory explain genotypically enhanced Mn tolerance in cowpea (Horst et al., 1999).

The presented work focuses on the characterization of H_2O_2-producing (NADH-*per*oxidase) and H_2O_2-consuming peroxidase (guaiacol-POD) in the apoplastic washing fluid (AWF) of leaves of cowpea cultivars differing in Mn tolerance and the factors affecting the resulting H_2O_2 formation. In order to get a more complete understanding of the Mn-induced changes in protein composition of the leaf AWF, two-dimensional electrophoresis resolutions of total soluble proteins of the leaf AWF from two cowpea cultivars differing in Mn tolerance were compared. Since of phenolic compounds were expected to be decisive in the development or avoidance of Mn toxicity, the effect of Mn excess on phenol concentrations and phenol composition in the AWF was investigated.

MATERIAL AND METHODS

Plant material and cultivation

Plants of cowpea (*Vigna unguiculata* (L.) Walp.) cultivars TVu 91 and TVu 1987 were grown hydroponically in a growth chamber under controlled environmental conditions at

30/25°C day/night temperature, 75±5% relative humidity and a photon flux density of 270 µmol m^{-1} s^{-1} photosynthetic active radiation at mid-plant height during a 16 h photoperiod. After germination in 1 mM $CaSO_4$, one seedling per cultivar was transferred to a constantly aerated nutrient solution in a 5 L plastic pot. The concentration of the basic nutrient solution was [µM]: $Ca(NO_3)$ 1000, KH_2PO_4 100, $MgSO_4$ 325, FeEDDHA 20, NaCl 10, H_3BO_3 8, $MnSO_4$ 0.2, $CuSO_4$ 0.2, $ZnSO_4$ 0.2, Na_2MoO_4 0.05.
After preculture for at least 14 days, the $MnSO_4$ concentration in the nutrient solution was increased up to 50 and 100 µM Mn, respectively. Control plants received 0.2 µM Mn continuously. Plants were treated until distinct visible symptoms were detectable on leaves of the sensitive cultivar. All plants were harvested at the same day.
The nutrient solution was changed two to three times a week to avoid nutrient deficiencies.

Quantification of Mn toxicity symptoms

For the quantification of Mn toxicity symptoms, leaf discs (1.54 cm^2) were cut out at the base, middle and tip of the trifoliate leaf and incubated in EtOH for at least 3 days. Numbers of brown spots per leaf disc were counted. Density of brown spots per cm^2 was calculated.

Extraction of Apoplastic Washing Fluid (AWF)

Apoplastic washing fluid (AWF) was extracted by a vacuum infiltration/centrifugation technique. Therefore, leaflets of second trifoliate leaves were cut from plants, weighted and infiltrated with demineralised water. The pressure was reduced to 35 hPa (1 min) by using a water jet pump followed by slow relaxation for 2 min carried out infiltration. Leaf lets were removed from the water and dry blotted. Infiltrated leaflets were weighted again and AWF was recovered by centrifugation at 1324 g for 5 min at room temperature.

Determination of protein concentration in the AWF

Measurements were carried out according to Bradford (1976).

Protein precipitation and 2D IEF/SDS-PAGE

Proteins from the AWF were extracted by phenol and precipitated by acetate/methanol (Fecht-Christoffers et al., 2003b).

For separation of proteins by their isoelectric point, the IPGphor system (Amersham Pharmacia Biotech, Uppsala, Sweden) was used. Proteins were resuspended in demineralised water and supplemented with a rehydration solution [8M urea, 2% (w/v) CHAPS, 0.5% (v/v) carrier ampholyte mixture (IPG buffer; Amersham Pharmacia), 0.28% (w/v) DTT and a trace of bromphenol blue. Focussing was carried out availing Immobiline DryStrip gels (18 cm) with a nonlinar pH gradient (pH 3-10) under conditions outlined by Werhahn and Braun (2002). Afterwards the Immobiline DryStrip gels were incubated with equilibration solution [50 mM Tris-Cl (pH 8.8), 6M urea, 30% (v/v) glycerin, 2% (w/v) SDS, bromphenol blue] supplemented with (i) 1% (w/v) DTT and (ii) 2.5% (w/v) iodoacetamide each for 15 min, respectively. DryStrip gels were placed horizontally on a Tricine SDS-PAGE. The second dimension electrophoresis was carried out according to Schägger and von Jagow (1987).

Staining

2D gels were stained with colloidal Coomassie-blue (Neuhoff et al., 1985; 1990). Gels were fixed in 40% (v/v) MeOH/10% (v/v) HAc for at least 2 h and incubated in 80% (v/v) staining solution and 20% (v/v) MeOH for at least 3 h. The staining solution contained 98% (v/v) solution A [2% (w/v) phosphoric acid, 10% (w/v) ammonia sulfate] and 2% (v/v) solution B [5% (w/v) coomassie blue].

Determination of guaiacol-POD activity

For measurement of H_2O_2-consuming POD activities in the AWF, the oxidation of the artificial substrate guaiacol was determined spectrophotometrically at λ=470 nm (UVIKON 943, BioTek Instruments GmbH, Neufahrn, Germany). Samples were mixed with guaiacol solution (20 mM guaiacol in 10 mM Na_2HPO_4 buffer (pH 6)) and 0.03% H_2O_2.

Determination of NADH-per*oxidase activity and its interference by Mn and p-coumaric acid*

For measurement of NADH-*per*oxidase activity, samples were combined with 0.3 mM NADH, p-coumaric acid and $MnCl_2$, whereas concentration of added p-coumaric acid was varied from 0 to 1.6 mM and $MnCl_2$ from 0 to 160 mM. All components were dissolved in 100 mM sodiumacetate buffer, pH 5. The activity was measured at λ=340 nm using a

microplate reader (µQuant, BioTek Instruments GmbH, Neufahrn, Germany). For calculation of enzyme activities, molar extinction coefficients were calculated at each p-coumaric concentration: for 0, 0.016, 0.16 and 1.6 mM p-coumaric acid ε= 4.9423, 4.6825, 4.6011 and 4.2378 mM^{-1}.

*Determination of the potential H_2O_2 formation by NADH-*per*oxidase in the apoplast*

The detection of H_2O_2 was based on the peroxidase-catalysed oxidation of guaiacol. AWF was used for the measurement of NADH-*per*oxidase activity (as described above). After at least 1 min detection of NADH oxidation 3 mM guaiacol (in 10 mM sodium phosphate buffer, pH 6) were added to the enzyme assay. Changes in absorption were monitored at λ=470 nm for 2 min. For calculation of H_2O_2 formation rate, the molar extinction coefficient 0.00378 μM^{-1} was used.

*Separation of proteins from the AWF and measurement of the effect of AWF filtrate on NADH-*per*oxidase activity*

To verify the effect of apoplastic water-soluble non-enzyme compounds of the apoplast on the activity of NADH-*per*oxidase, proteins of the AWF were separated from the AWF by mini centrifuge filter with a molecular weight cut-off (MWCO) at 4 kDa (Nalge Nunc International, Rochester, USA). Samples volumes of 500 µl were centrifuged at 6000 g, 4°C for 1-2 h. AWF filtrates were removed and protein concentrates were washed with double demineralised water (ddH_2O) at 4°C. Purified proteins were recovered by centrifugation at 3000 g, 4°C and 10 min.
Just before detection of enzyme activities, proteins and AWF filtrate of controls and Mn treated plants from TVu 91 and TVu 1987 were crosswise combined. For measurement of enzyme activities, 1 µl of protein concentrate and 50 µl AWF filtrate or ddH_2O, respectively, were mixed with 16 mM $MnCl_2$, 1.6 mM p-coumaric acid, and 0.3 mM NADH.

Measurement of total phenol concentration in the AWF and separation of phenols by HPLC

AWF (50 µl) was combined with 350 µl ddH_2O and 50 µl Folin-Denis solution (Merck, Darmstadt, Germany). After 3 min incubation 100 µl saturated Na_2CO_3 solution was added. After 1 h incubation and centrifugation (1 min, 5000 x g) absorption was measured

at λ=725 nm in a microplate reader (μQuant, BioTek Instruments GmbH, Neufahrn, Germany). Concentrations were calculated in relation to p-coumaric acid standard solutions.

Since most water-soluble phenolics in plant leaves are glycosides (Harborne, 1977), for the separation and identification of the phenolics the glucosides were hydrolysed with HCl. AWF (4 ml) was hydrolysed in the presence of HCl (0.37 M) and 100 µl acetonitril (ACN). After 1h incubation at 80°C, 66 µl of saturated $NaHCO_3$ and 100 µl Acetonitril were added. Samples were loaded on Bankerbond spe^{TM} octadecyl C18 columns (Mallinckrodt Baker, Phillipsburg, USA), which were previously equilibrated with methanol and ddH_2O. After loading, columns were washed with ddH_2O and samples were eluted with 500 µl ACN. The acidic hydrolysation followed by solid phase extraction using C18-columns provided recovery rates of 90 to 95%, approximately. In contrast, phenolic compounds were strongly affected by hydrolysation with NaOH. The extraction with ethyl acetate resulted in low recovery rates (results not shown).

HPLC analyis was carried with a HPLC-DAD system (BioTek Instruments GmbH, Neufahrn, Germany; pre-column: Phenomenex AJ0-4286 C18; column: Phenomenex Luna 5µ C18(2) (Phenomenex, Aschaffenburg, Germany). The mobile phase used was (A) ACN and (B) H_3PO_4 [0.1% (w/v)]. The solvent gradient changed as follows: 1) time 0-5 min, 0% A/100% B, flow 1 ml min^{-1}; 2) time 5-12 min, 16% A/84% B, flow 0.5 ml min^{-1}; 3) time 12-45 min, 25% A/75% B, flow 1 ml min^{-1}; 4) time 45-50 min, 100% A/0% B, flow 1 ml min^{-1}; 5) time 50-65 min, 0% A/100% B, 1 ml min^{-1}. Absorptions were measured at λ=230, 266, 280 and 320 nm. Calibarations were carried out with p-hydroxybenzoic acid, p-coumaric acid, o-coumaric acid, chlorogenic acid, ferulic acid, synapinic acid, p-hydroxybenzaldehyde, catechin, salicylhydroxamic acid, salicylic acid, benzoic acid, vanillic acid, syringic acid, caffeic acid, syringaldehyde, protocatechenic acid, tannic acid, gentisic acid and gallic acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Mineral analysis

For detection of Mn in the bulk-leaf tissue centre ribs were cut out of the middle leaflets of trifoliate leaves and 0.5 – 1 g leaf tissue were dried at 65°C till constant weight. Drying was followed by dry ashing (480°C, 8h) and dissolving the ash in 6 M HCl with 1.5% (w/v) hydroxylammonium chloride and dilution (1:10) with ddH_2O. AWF was diluted 1:10, whereas HCl and hydroxylammonium chloride was added to give final concentration

of 0.6 M HCl and 0.15% (w/v) hydroxylammoniumchloride. Measurements were carried out by optical emission spectrometry, inductive coupled plasma (Spektro Flame, Spectro, Kleve, Germany).

Statistical analysis

Means and standard deviations were from independent replications within an experiment and calculated by Excel 2000 (Microsoft Corporation, USA). The numbers of replications are noted in figure legends. Regression analysis, analysis of variance and multiple comparisons of means (Tukey test) were carried out by SAS 8e (SAS Institute Inc., Cary, North Carolina, USA). Levels of significance are given in graphs by *, **, *** for $p< 0.05$, 0.01, 0.001. Different letters are significantly different at $p<0.05$ (Tukey).

RESULTS

To investigate physiological effects of Mn stress on cowpea, two previously described cultivars (TVu 91 and TVu 1987, Horst et al., 1999), which differ considerably in Mn tissue tolerance, were compared under various conditions:

Manganese uptake and formation of visible Mn toxicity symptoms

Plants of cowpea cultivars TVu 91 and TVu 1987 readily took up manganese, thus causing 10-fold higher Mn contents in the leaf tissue than of control plants. Characteristic Mn toxicity symptoms (brown spots) were expressed on leaves of cv TVu 91, whereas cv TVu 1987 was nearly unaffected (Fig. 1A). In spite of similar Mn tissue contents, cv TVu 1987 showed significantly higher Mn concentration in the AWF than cv TVu 91 (Fig. 1B).

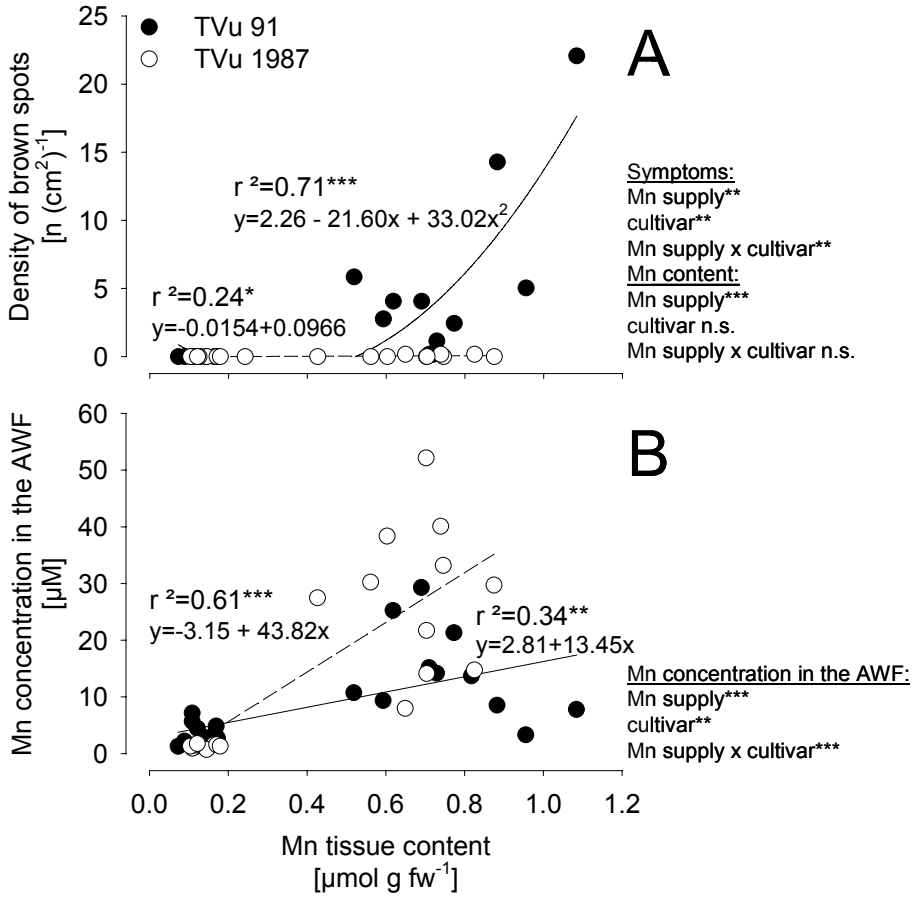

Figure 1 Relationship between Mn content in the bulk leaf tissue and (A) the density of brown spots on leaves and (B) the Mn concentration in the AWF. Plants of cowpea cultivars TVu 91 and TVu 1987 were treated with 50 µM Mn for 4 days, whereas control plants received 0.2 µM Mn continuously. n=12. Levels of significance are shown by *, **, *** for p<0.05, 0.01, 0.001.

Effect of Mn treatment on the protein composition in the AWF

2D resolution of AWF proteins extracted from leaves of cv TVu 91 treated with elevated Mn concentration showed enhanced expression of proteins in the acid (pH 3-4) and neutral (pH 6) range of the polyacrylamide gel compared to control plants, whereas protein pattern of cv TVu 1987 was hardly affected by the Mn treatment. The 2D resultion from cv TVu 91 showed a high conformity to previously shown results. Therefore, strongly expressed proteins on the gels in Fig. 2 were considered identical with proteins identified previously by nanoLC-MS/MS (Fecht-Christoffers et al., 2003b), e.g. pathogenesis related like proteins class I (spot 2, 8 and 10), chitinase (spot 13, 14 and 16), peroxidase (spot 16 and 18) and glucanase (spot 18 and 20).

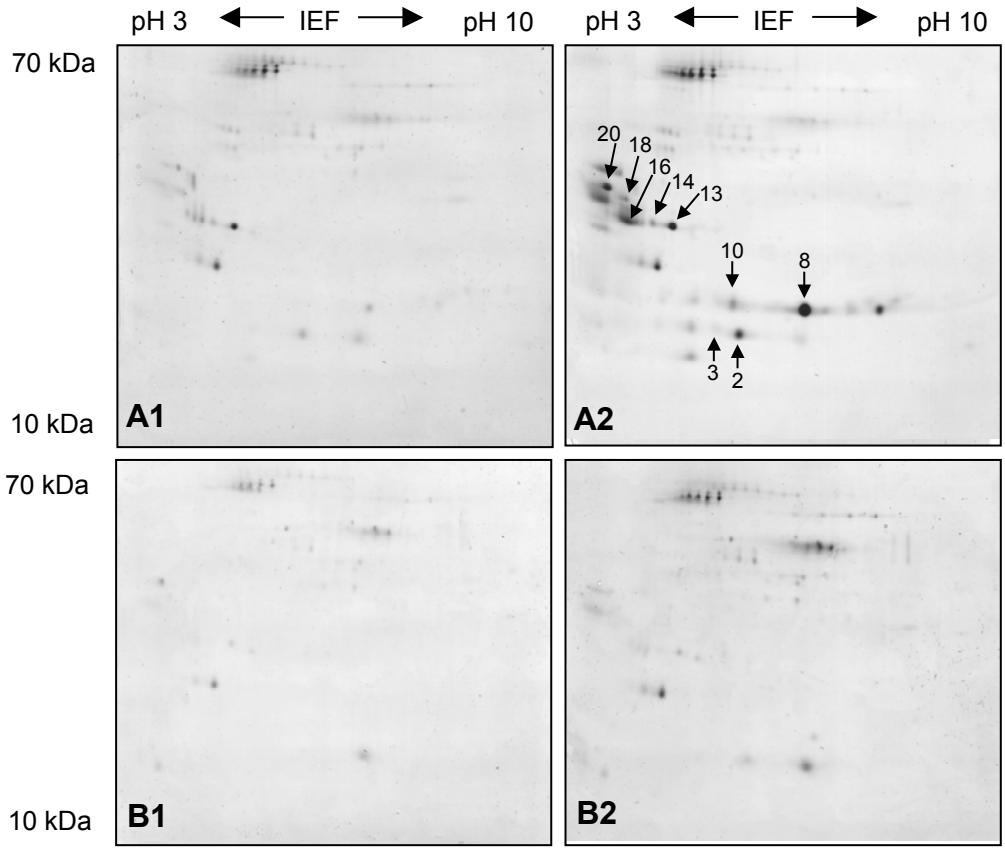

Figure 2 2D resolution of water-soluble proteins from the leaf apoplast of the two cowpea cultivars TVu 91 (A1/A2) and TVu 1987 (B1/B2) differing in Mn tolerance by IEF/SDS-PAGE. Plants precultured for 13 days were treated with 50 µM Mn for 5 days (A2, B2), while control plants received 0.2 µM Mn continuously (A1, B1). Numbers on the top of the gels indicate the pH gradient, and the numbers on the left indicate molecular masses of standard proteins. Arrows indicate identified proteins (the numbers correspond to proteins identified in Fecht-Christoffers et al., 2003b).

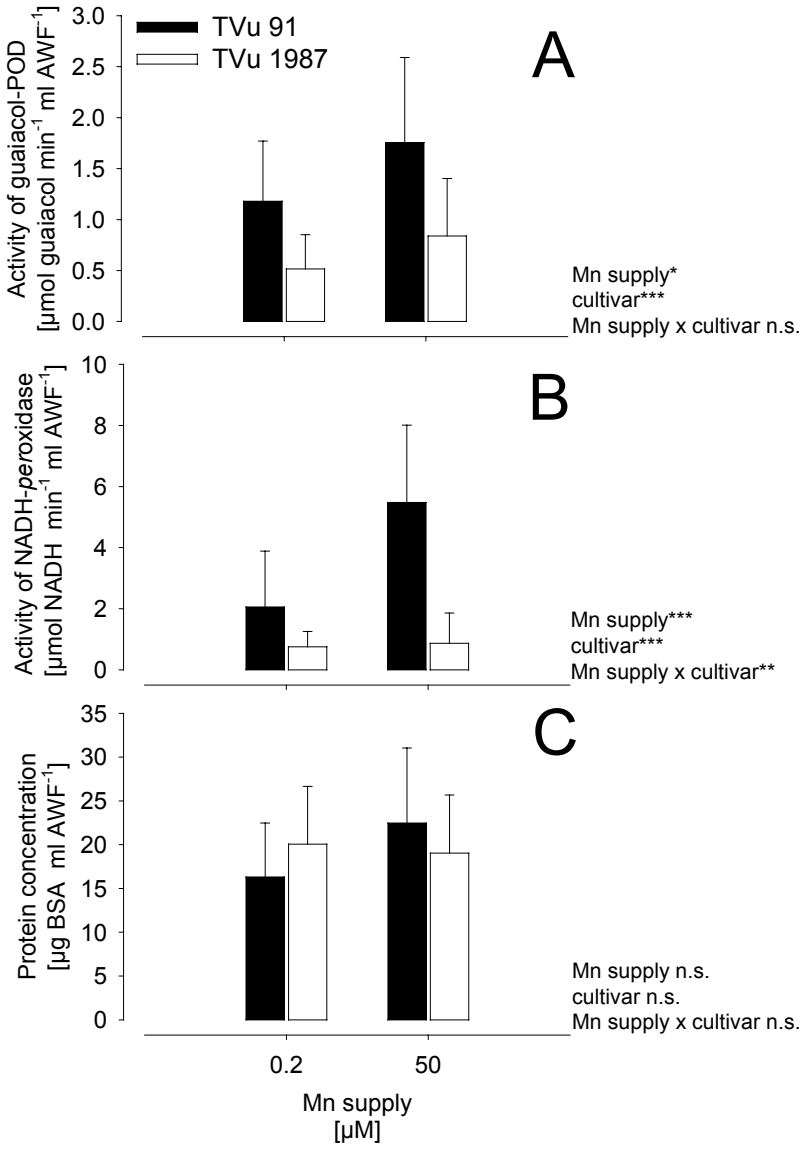

Figure 3 Effect of excess Mn on the activity of (A) guaiacol-POD, (B) NADH-*per*oxidase, and (C) the protein concentrations in the AWF extracted from leaves of cowpea cultivar TVu 91 and TVu 1987. Plants were treated with 50 µM Mn for 4 days, whereas control plants received 0.2 µM Mn continuously. n=12. Levels of significance are shown by *, **, *** for p<0.05, 0.01, 0.001.

*Effect of Mn treatment on activities of guaiacol-POD, NADH-*per*oxidase and the protein concentration in the AWF*

Mn treatment induced an increase of apoplastic guaiacol-POD and NADH-*per*oxidase in both cultivars (Fig. 3). However, this effect was only significant for NADH-*per*oxidase in cv TVu 91. Enzyme activities in the AWF of cv TVu 1987 were generally significantly lower than in cv TVu 91. The activities presented are representative for the specific activities because the protein concentrations of the AWF did not significantly differ between cultivars and Mn-treatments (Fig. 3C). There was a tendency of Mn treatment-

enhanced protein concentrations in the AWF of cv TVu 91. A significant positive correlation existed between guaiacol-POD and NADH-*per*oxidase in both cultivars. However, the NADH-*per*oxidase activity was higher in cv TVu 91 at comparable elevated guaiacol-POD activities (Fig. 4).

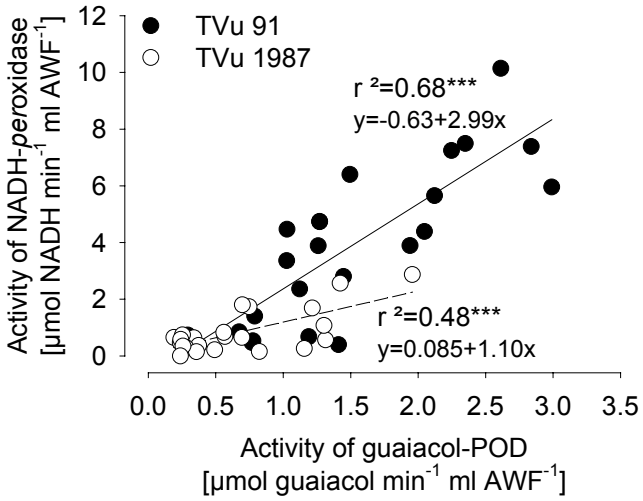

Figure 4 Relationship between the activity of guaiacol-POD and NADH-*per*oxidase in the AWF extracted from leaves of cowpea cultivars TVu 91 and TVu 1987. Plants were treated with 50 µM Mn, whereas control plants received 0.2 µM Mn continuously. n=12. Levels of significance are shown by *, **, *** for p<0.05, 0.01, 0.001.

*The formation of H_2O_2 by leaf AWF NADH-*per*oxidase in vitro*

The NADH-*per*oxidase mediated formation of H_2O_2 (*in vitro*) was significantly lower in the leaf AWF of cv TVu 1987 than in cv TVu 91 (Fig. 5). H_2O_2 formation was significantly enhanced by Mn treatment in cv TVu 91 but not in cv TVu 1987. The H_2O_2 formation significantly correlated positively with the NADH-*per*oxidase activity in both cowpea cultivars. But both, H_2O_2 formation rate and NADH-*per*oxidase activity were significantly higher in cv TVu 91 than in cv TVu 1987 (Fig. 6).

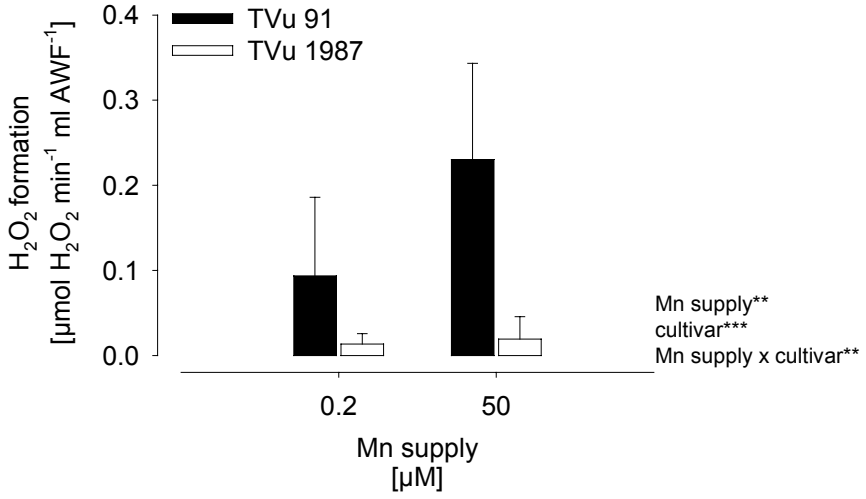

Figure 5 *In vitro* formation of H_2O_2 by AWF proteins extracted from leaves of cowpea cultivars TVu 91 and TVu 1987 differing in Mn tolerance as affected by Mn supply during cultivation of plants. Plants were treated with 50 µM Mn, whereas control plants received 0.2 µM Mn continuously. n=12. Levels of significance are shown by *, **, *** for p<0.05, 0.01, 0.001.

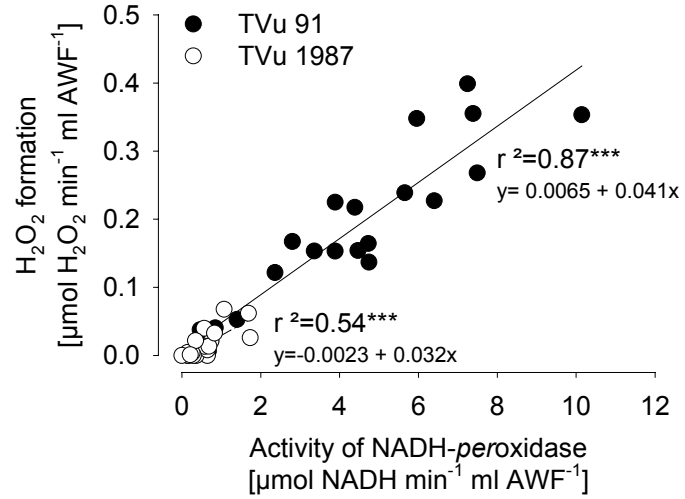

Figure 6 Relationship between the activity of NADH-*per*oxidase and the H_2O_2 formation *in vitro* by AWF proteins extracted from leaves of cvs TVu 91 (Mn sensitive) and TVu 1987 (Mn tolerant). Plants were treated with 50 µM Mn, whereas control plants received 0.2 µM Mn continuously. Measurements of NADH-*per*oxidase activity were carried out first, followed by the measurement of H_2O_2 formation. n=12. Levels of significance are shown by *, **, *** for p<0.05, 0.01, 0.001.

Effect of Mn and p-coumaric acid on the activity of NADH-peroxidase from AWF and potential H_2O_2 formation

The NADH oxidation rate by NADH-*per*oxidase was significantly affected by the concentration of p-coumaric acid and Mn *in vitro* (Fig. 7). Enzyme activities increased with increasing concentration of p-coumaric acid up to 1.6 mM and increasing Mn concentration up to 1.6 mM. The application of 16 mM Mn did not further enhance NADH-*per*oxidase activity and the highest Mn concentration applied (160 mM) strongly suppressed the NADH oxidation rate. AWF from plants treated with 50 µM Mn during cultivation showed generally higher NADH-*per*oxidase activities than control plants. This was observed in both cultivars. However, NADH-*per*oxidase activity was significantly lower in the Mn-tolerant cv TVu 1987 than in the Mn-sensitive cv TVu 91.

In agreement with the NADH-*per*oxidase activity, the formation rate of H_2O_2 *in vitro* was similarly dependent on the presence of p-coumaric acid and Mn (Fig. 8). The highest H_2O_2 formation rate was measured in the presence of 1.6 mM p-coumaric acid and 1.6 mM Mn. Application of 16 mM Mn did not further enhance H_2O_2 formation in cv TVu 91 but was supraoptimal in cv TVu 1987. In contrast to NADH-*per*oxidase activity, H_2O_2 formation did not significantly differ between the cultivars.

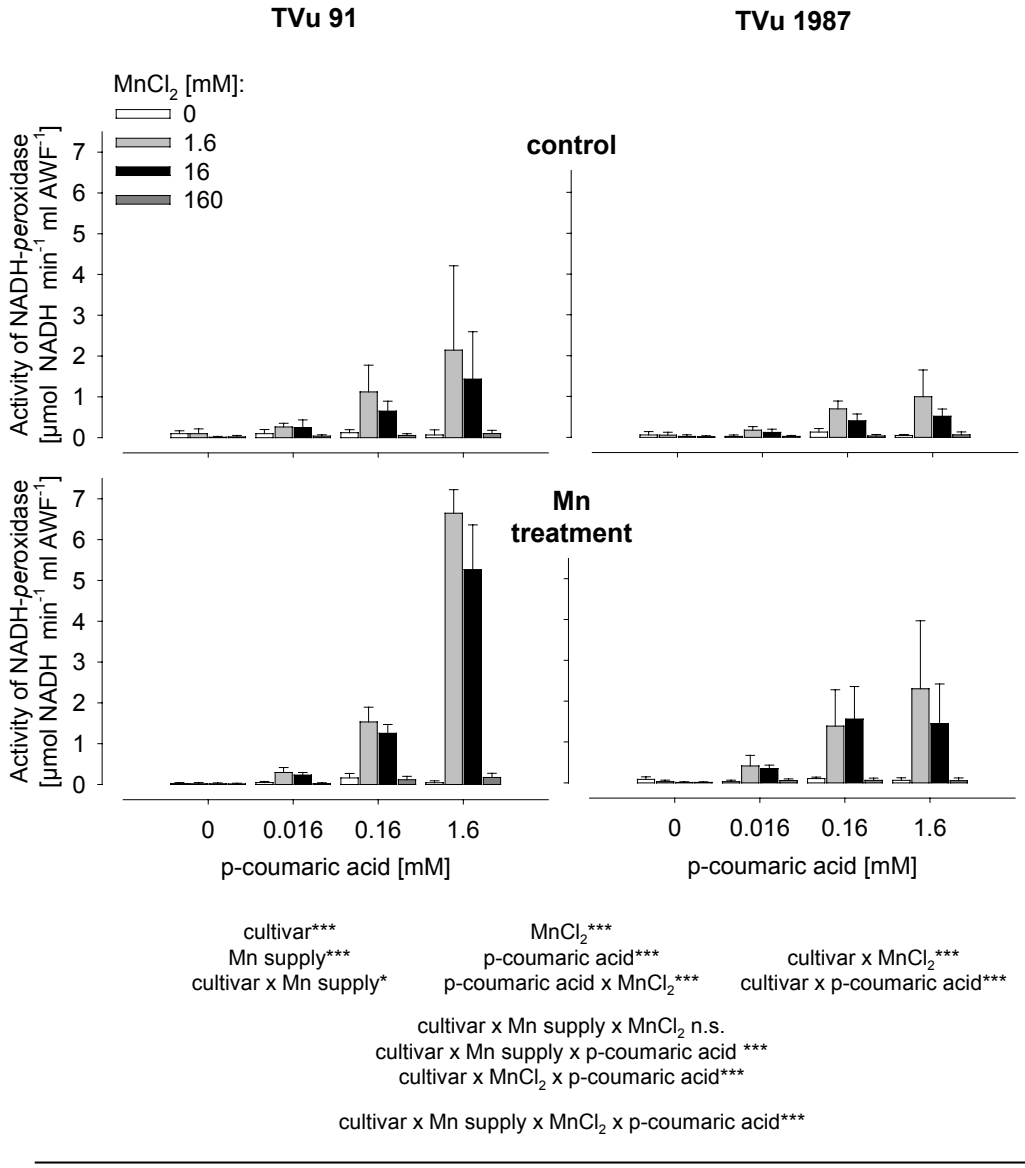

Figure 7 Effect of $MnCl_2$ and p-coumaric acid concentrations *in vitro* on activity of NADH-*per*oxidase in the AWF extracted from leaves of controls and Mn treated plants (50 µM Mn) of cowpea cultivars TVu 91 (Mn sensitive) and TVu 1987 (Mn tolerant). Concentration of added p-coumaric acid varied from 0 to 1.6 mM and $MnCl_2$ from 0 to 160 mM. n=4. Levels of significance are shown by *, **, *** for $p<0.05$, 0.01, 0.001.

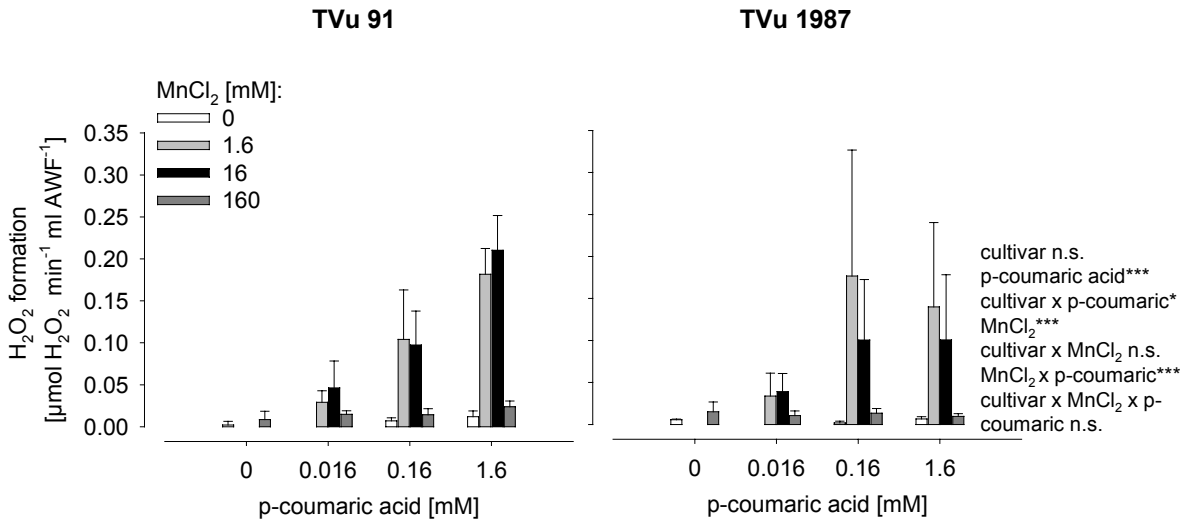

Figure 8 Effect of $MnCl_2$ and p-coumaric acid concentration *in vitro* on the formation rate of H_2O_2 by AWF proteins from Mn treated plants (50 µM Mn) of cowpea cultivars TVu 91 and TVu 1987. Concentrations of added p-coumaric acid varied from 0 to 1.6 mM, and of $MnCl_2$ from 0 to 160 mM. n=4. Levels of significance are shown by*, **, *** for p<0.05, 0.01, 0.001.

*Effect of apoplastic water-soluble metabolites on the NADH-*per*oxidase activity in the AWF*

To investigate the influence of water-soluble metabolites in the AWF on the NADH-*per*oxidase activity, the purified apoplastic proteins from controls and Mn treatments of both cultivars were crosswise combined with protein-free AWF (AWF filtrate) or water. AWF filtrate alone and boiled samples showed no NADH-*per*oxidase activities (data not shown). The AWF filtrates added to the enzyme assay had differential effects on the kinetics of NADH oxidation (Fig. 9). In the presence of AWF filtrates from cv TVu 91 or water, the NADH oxidation by apoplastic enzymes of cv TVu 91 and cv TVu 1987 (controls and Mn treatments) started shortly after addition of NADH (lines a, b, and e in each plot). AWF filtrates from cv TVu 91 accelerated the oxidation compared to water. In the presence of AWF filtrate from cv TVu 1987 (lines c and d in each plot), NADH oxidation showed a lag phase of 2 to 8 minutes. The longest lag phase was observed in the combination of both protein and filtrate of cv TVu 1987 treated with high Mn (Dd). The combination of proteins from cv TVu 1987 and water (Ce, De) also produced a short lag phase.

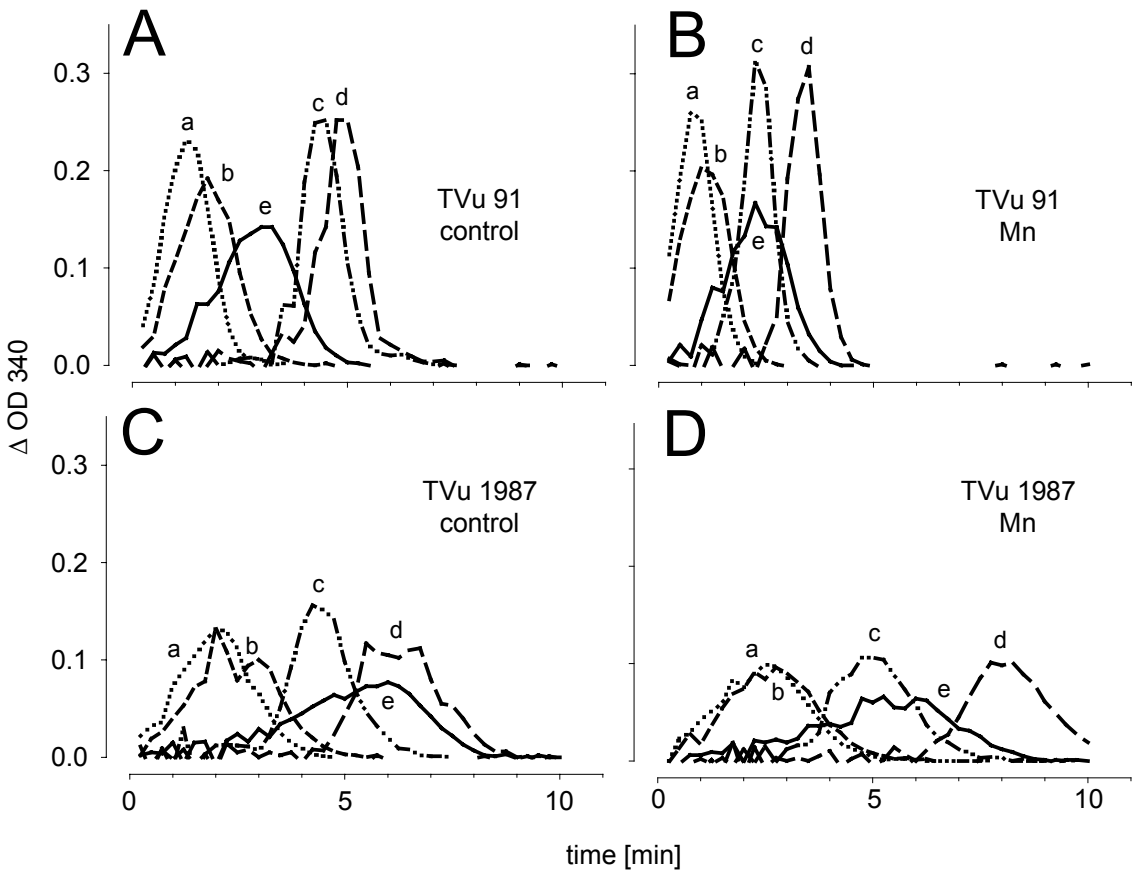

Figure 9 Effect of water-soluble apoplastic metabolites (AWF filtrate) or water on the consumption of NADH by purified apoplastic proteins. Oxidation of NADH is displayed as changes in absorption at λ=340 nm. AWFs from controls and Mn treatments from cvs TVu 91 and TVu 1987 were divided in two fractions (purified proteins and AWF filtrate) by using centrifugal concentrators. Protein concentrates, AWF filtrates (a, TVu 91 control; b, TVu 91 Mn; c, TVu 1987 control; d, TVu 1987 Mn) and water (e) were combined immediately before addition of NADH.

From the kinetics of NADH oxidation, the duration of the lag phase and the slope at maximum NADH-oxidation rate (max V) was calculated and submitted to statistical analysis (Fig. 10). The lag phase (Fig. 10A) was significantly affected by the origin of AWF filtrate, the origin of the AWF protein, and the Mn status of the plants: AWF filtrates from leaves of cv TVu 91 reduced the lag phase particularly in combination with AWF proteins from the same Mn-treated cultivar. Filtrates from TVu 1987 delayed the NADH oxidation especially of proteins from the same Mn-treated cultivar. No significant effect of the origin of the AWF filtrates on the maximum NADH-oxidation rate existed (Fig. 10B). However, AWF proteins from cv TVu 91 generally, but more clearly when Mn-treated, showed a significantly higher NADH-oxidation capacity than proteins form cv TVu 1987.

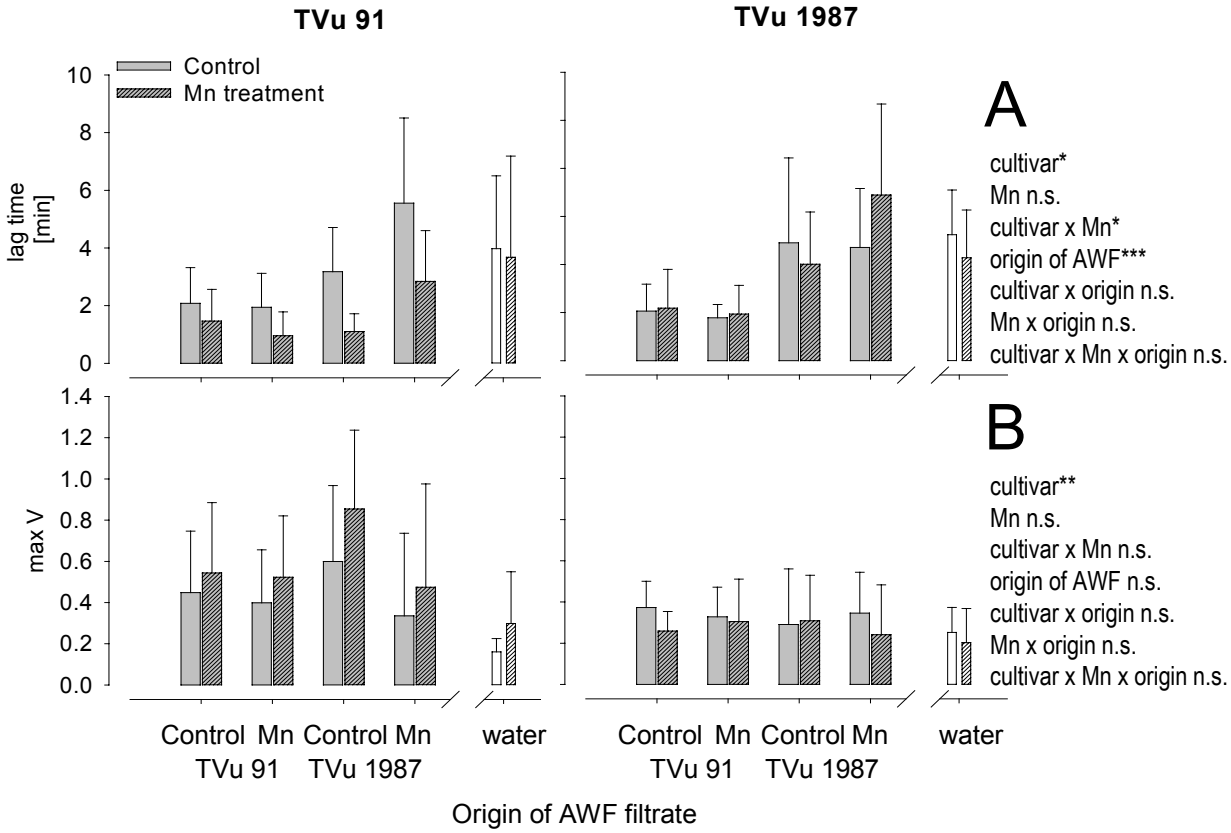

Figure 10 Effect of the origin of protein-free AWF filtrate and water on the functionality of purified NADH-*per*oxidase from the AWF extracted from leaves of cowpea cultivars TVu 91 (Mn sensitive) and TVu 1987 (Mn tolerant). Time course of NADH oxidation is characterized by (A) the lag time of delayed NADH oxidation and (B) the slope of NADH oxidation at maximum oxidation rate (max V). AWF from controls and Mn treatments of cowpea plants were divided in two fractions (purified proteins and protein-free AWF filtrate) by using centrifugal concentrators with MWCO 4kDa. Proteins, AWF filtrates and water were combined immediately before detection of NADH-*per*oxidase activity. n=6. Levels of significance are shown by *, **, *** for $p<0.05$, 0.01, 0.001.

Concentrations and composition of phenolic compounds in the AWF

In spite of considerable variability, cowpea cultivar TVu 91 showed significant higher phenol concentration in the AWF than cv TVu 1987 (Fig. 11). Kinetic experiments with cv TVu 91 demonstrated a release of phenols into the apoplast with increasing severity of Mn toxicity (Fig. 12). In leaves of cv TVu 91 with no or slightly expressed Mn toxicity symptoms (density of brown spots 0 to 35 per cm^2), apoplastic phenol concentrations did not differ significantly. In leaves with spot densities of at least 50 per cm^2, phenol concentrations in the AWF were significantly increased.

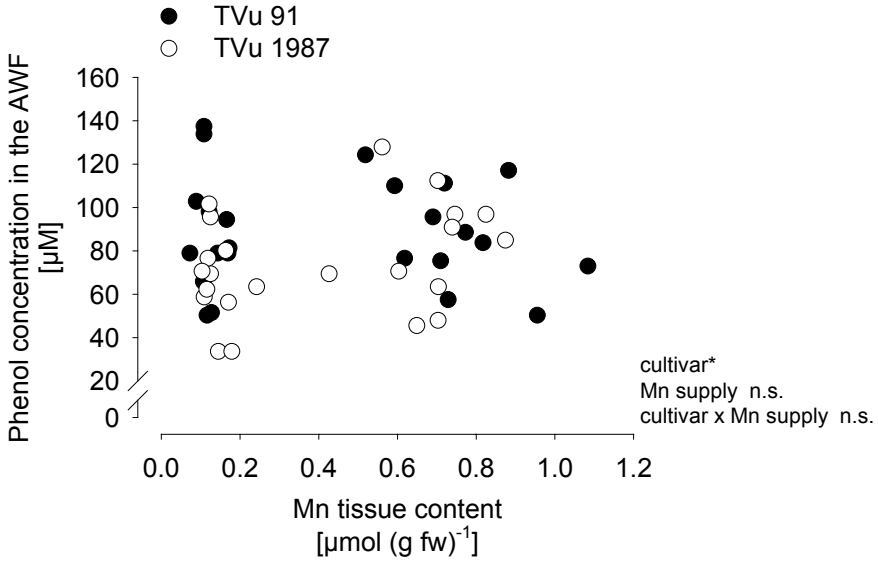

Figure 11 Relationship between Mn tissue content and the phenol concentration in the leaf AWF of two cowpea cultivars TVu 91 and TVu 1987, differing in Mn tolerance. Plants were treated with 50 µM Mn for 4 days, whereas control plants received 0.2 µM Mn continuously. Phenol concentrations were calculated using standard solutions of p-coumaric acid in the range from 0 to 250 µM. n=12. Levels of significance are shown by *, **, *** for p<0.05, 0.01, 0.001.

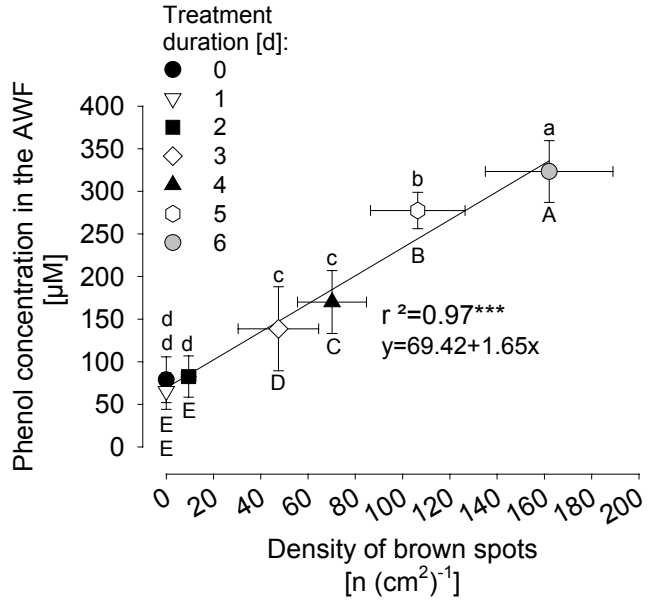

Figure 12 Relationship between the phenol concentration in the AWF and the density of brown spots. Plants of cowpea cultivar TVu 91 were precultured in nutrient solution and Mn concentration was increased for 1, 2, 3, 4, 5, or 6 days, respectively. Control plants received 0.2 µM continuously. n=14 . Significant differences between mean values are indicated by different letters at p<0.05 (Tukey): capital letters for density of brown spots; small letters for phenol concentrations. Levels of significance are shown by *, **, *** for p<0.05, 0.01, 0.001.

The significant increase of the total phenol concentration in the AWF of cv TVu 91 (Fig. 12) with advanced expression of Mn toxicity symptoms was reflected in increased peak hights, but also in the appearance of additional peaks in the chromatogram (Fig. 13B). Differences in phenol compositions exist between control plants of the cultivars TVu 91 and TVu 1987 (Fig. 13A). The AWF from cv TVu 1987 showed at least 4 additional peaks in the chromatogram. Since no similarities between absorptions spectra of apoplastic and commercial available phenolic compounds existed, the identities of the phenolic compounds in the AWF remain unknown so far.

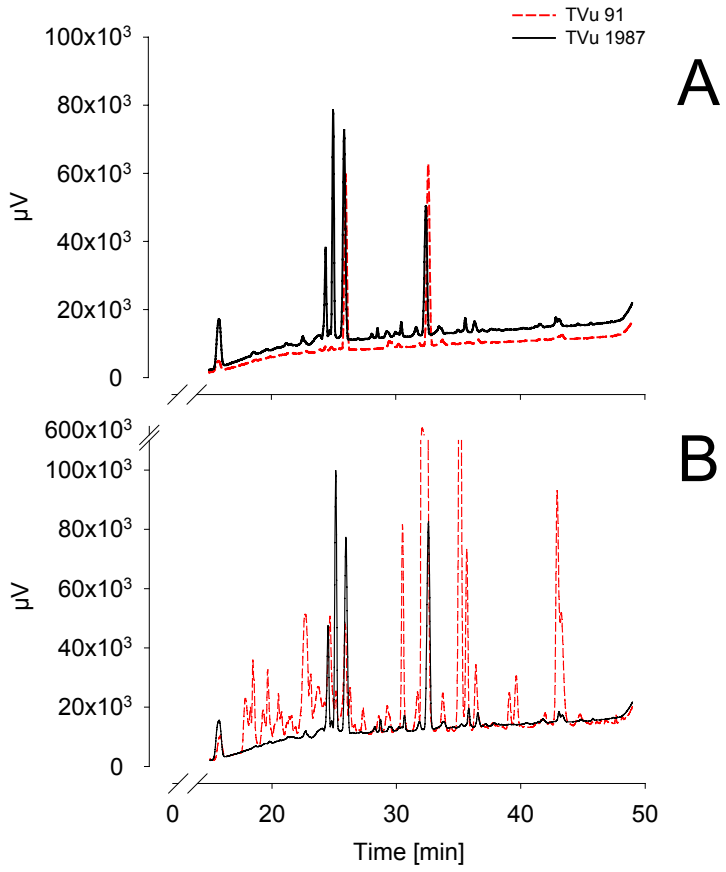

Figure 13 Chromatograms of phenols in the AWF from the leaves of (A) controls and (B) Mn treatments of cowpea cultivars TVu 91 and TVu 1987. Plants were treated with 100 µM Mn for 4 days, whereas control plants received 0.2 µM Mn continuously. AWF were acidic hydrolysed at 80°C and phenols extracted by solid phase extraction.

DISCUSSION

Mn uptake and development of Mn toxicity symptoms

Excess manganese supply during the cultivation of cowpea plants caused the formation of visible small dark-brown spots on leaves, representing oxidized Mn and phenols (Wissemeier and Horst, 1992). The density of these brown spots is a reliable parameter for the severity of Mn toxicity (Wissemeier and Horst, 1991). In the present work, the formation of 5 – 10 brown spots per cm^2 leaf area (Fig. 1) represents an early and moderate stage of Mn toxicity without further distinct Mn toxicity symptoms, such as chlorosis and necrosis. As previously shown (Horst et al. 1999; Fecht-Christoffers et al., 2003a), cowpea cultivars TVu 91 and TVu 1987 show significant differences in the expression of Mn toxicity symptoms at elevated Mn tissue contents. Therefore, the Mn-resistance of TVu 1987 is due to the ability to tolerate high Mn concentrations in the leaf tissue. The appearance of symptoms is accompanied by a steep increase of the Mn concentration of the AWF indicating a particular role of free apoplastic Mn for the expression of Mn toxicity in spite of its low contribution to the total Mn content of the leaf (Fecht-Christoffers et al., 2003b). The significantly higher Mn concentration in the AWF of leaves of Mn-treated plants of TVu 1987 compared to TVu 91 (Fig. 1) demands for a particularly efficient Mn-tolerance mechanism in this cultivar.

The apoplast proteome: 2D IEF/SDS-PAGE resolutions of proteins in the AWF from cultivars differing in Mn tolerance

In the Mn-sensitive cultivar TVu 91, development of toxicity symptoms is accompanied by an enhanced release of proteins into the leaf apoplast (Fecht-Christoffers et al., 2003b). The protein concentration in the AWF is dependent on the severity of Mn toxicity symptoms. The only slight and non-significant increase of the apoplastic protein concentration (Fig. 3C) reflects the mild expression of Mn toxicity in this experiment. The 2D resultions of water-soluble apoplastic proteins from cv TVu 91 (Fig. 2) in this study was carried out with AWF from leaves with a stronger expression of Mn toxicity (density of brown spots higher than 30 per cm^2). The 2D resolution showed high conformity to previously shown results (Fecht-Christoffers et al., 2003b). Therefore, strongly expressed leaf AWF proteins in Mn-treated cv TVu 91 presented (Fig. 2) were most probably identical with the proteins identified previously by nanoLC-MS/MS (Fecht-Christoffers et

al., 2003b). Among the most prominently expressed proteins were pathogenesis-related proteins class I, chitinase, peroxidase, and glucanase. The role of the pathogenesis-related like proteins in Mn toxicity is not clear (Fecht-Christoffers et al., 2003b). 2D resolution of proteins in the AWF extracted from Mn-tolerant TVu 1987 did not show Mn-induced changes of the apoplast proteome. This confirms the assumption that the release of proteins in the Mn-sensitive cultivar reflects Mn sensitivity rather than a Mn-tolerance mechanism (Fecht-Christoffers et al., 2003b). However, it has to be considered that minor changes in the apoplast proteome could not be detected due to a too low amount of proteins loaded on the gel. Therefore, it cannot yet be excluded that apoplastic proteins are involved in the enhanced Mn tolerance of TVu 1987. Also, only water-soluble proteins of the leaf apoplast have been analysed so far.

PODs in the leaf apoplast and their role in Mn toxicity

Plant peroxidase isoenzymes (EC 1.11.1.7) are monomeric heme-containing enzymes with differences in molecular mass, isoelectric point, pH optima, substrate specificity, function, and localization within the plant tissue (Campa, 1991). Particular basic (cationic) POD isoenzymes, present in young and developing tissues (MacAdam et al., 1992b; Polle et al., 1994) are attributed to play a role in the production of H_2O_2 in the cell wall (Mäder et al., 1980; Mäder and Amberg-Fisher, 1982). Because NADH was shown to act as electron donor, H_2O_2-producing POD was also named `NADH oxidase´. Apoplastic acidic (anionic) PODs were frequently detected in maturing tissues (MacAdam et al., 1992; Polle et al., 1994; de Souza and MacAdam, 1998; Klotz et al., 1998) and showed high affinities to H_2O_2 and monophenolic compounds (Mäder et al., 1977; Mäder et al., 1980). Therefore, they are considered to be involved in secondary cell-wall formation and lignification by reduction of H_2O_2 and oxidation of phenolic compounds (Mäder et al., 1980; Mäder et al., 1986; Lagrimini et al., 1993; Ros Barcelo, 1997). Beside the discrimination of POD isoforms according to their functionality, PODs were also proposed to act as polyfunctional enzymes (Pedreño et al., 1995). Such peroxidases undergo at least two reaction cycles (Yamazaki and Yokota, 1973; Pedreño et al., 1995). The peroxidase-oxidase cycle is characterized by the formation of ferrous POD (Fe^{2+}) and the inactive compound III (oxyperoxidase Fe^{6+}) in the presence of NADH under aerobic conditions (Yamazaki and Yokota, 1973; Pedreño et al., 1987). This cycle was proposed to be the source of H_2O_2 formation (Halliwell, 1978). A two-electron transfer to H_2O_2, followed by

two single reduction steps back to the ferric (Fe^{3+}) enzyme, characterizes the peroxidatic reaction. This "classical" peroxidatic cycle represents probably the source of phenol oxidation and polymerisation in the cell wall. In cowpea, the magnitude of the POD activity is indicative of the severity of Mn toxicity (Fecht-Christoffers 2003a,b), like other indicators e.g. the formation of brown spots and of callose (Wissemeier and Horst, 1991; Wissemeier et al., 1992). It was demonstrated that Mn treatment induced a release of PODs into the apoplast, which was a main reason for the steep increase of apoplastic POD activity. The apoplastic washing fluid of cv TVu 91 contains several POD isoenzymes, which were not completely characterized up to now. Mn toxicity-released PODs are presumably acidic (anionic) POD-isoforms (Fig. 2; see Fecht-Christoffers et al., 2003b). The release of acidic isoenzymes was attributed to a late response to several stresses (Gaspar et al., 1985; Castillo, 1986), catalysing the "typical" H_2O_2-consuming peroxidatic cycle. Therefore, released acidic PODs probably catalyse the formation of brown depositions in the cell wall. Observations of Lagrimini (1991) support this hypothesis, because transgenic plants over-expressing acidic PODs showed more rapid wound-induced browning of tissues than control plants. The AWF showed also NADH-*per*oxidase activity, which was strongly activated by Mn treatment exclusively in the Mn-sensitive cv TVu 91 (Fig. 3B). Apoplastic guaiacol-POD isoenzymes of cowpea identified in the gel after BN-PAGE (Fecht-Christoffers et al., 2003b) showed also NADH-*per*oxidase activity (data not shown). Therefore, we conclude that apoplastic PODs in cowpea are able to act as polyfunctional enzymes. The stronger activation of NADH-*per*oxidase than guaiacol-POD (Fig. 3A, B and 4) suggests that the NADH-*per*oxidase activity is more directly involved in Mn toxicity than the H_2O_2-consuming function of POD.

*The effect of co-factors on NADH-*per*oxidase activity*

The regulatory effect of phenolic compounds and Mn on NADH-*per*oxidase activity is widely documented (Yamazaki and Piette, 1963; Halliwell, 1978; Mäder et al., 1980; Mäder and Füssl, 1982; Pedreño et al., 1987). As shown in Fig. 7, the concentrations of applied Mn and p-coumaric acid to apoplastic enzymes had a significant effect on NADH-*per*oxidation rate by AWF proteins. Maximum NADH-*per*oxidase activity was measured in the presence of 1.6 to 16 mM Mn, and 1.6 mM p-coumaric acid. This was true for both genotypes. Due to experimental reasons, the effect of p-coumaric acid concentrations higher than 1.6 mM was not tested. Therefore, a further stimulation of NADH-*per*oxidase

by higher phenol concentration cannot be excluded. Documented concentrations of added phenols ranged from 0.01 to 2.5 mM and the $MnCl_2$ concentration used varied from 0.1 to 50 mM (Mäder and Amberg-Fisher, 1982; Pedreño et al., 1987; Otter and Polle, 1997; Lin and Kao, 2001). Thus the selected p-coumaric and $MnCl_2$ concentrations in our experiments were in the upper but not supraoptimal range.

*The particular role of Mn in NADH-*per*oxidase activity*

Mn^{II} was proposed to affect NADH-*per*oxidase via reduction of $O_2^{-\cdot}$ or $HO_2^{\cdot}$ thus forming Mn^{III} (Yamazaki and Piette, 1963; Halliwell, 1978). Indeed, the beneficial property of Mn^{II} as scavenger for reactive oxygen species is documented (Kawano et al., 2002) and discussed as an SOD equivalent system in lactic acid bacteria (Archibald and Fridovich, 1982a, b). Therefore, the suppression of NADH-*per*oxidase in the presence of 160 mM $MnCl_2$ (Fig. 7) might by caused by a complete scavenging of reactive oxygen radicals (RORs) produced in the POD cycles. The scavenging of $O_2^{-\cdot}$ by Mn^{II} *in vitro* is significantly affected by the presence of several buffers and complexing agents. Aqueous Mn^{2+} proved to be a poor scavenger for reactive oxygen species (ROS), whereas in the presence of orthophosphate, pyrophosphate, lactate, succinate, and malate the ROS scavenging activity of Mn^{II} increased (Archibald and Fridovich, 1982a). The nature of the ligand determines the stability of the manganous complex. This is shown by ESR spectra from Mn-pyrophosphate complexes, which showed reduced amplitudes due to increased spin exchange between the unpaired d-orbital electrons of Mn^{II} and the ligand electrons. Polyphosphate was less effective, and only a slight stabilisation of Mn^{II} by SO_4^{2-} was observed. Already Kenten and Mann (1950) detected the formation of a stable manganous complex with pyrophosphate but not with orthophosphate. Apoplastic Mn concentrations up to 500 µM *in vivo* (Fig. 1) might stimulate NADH-*per*oxidase more effectively due to the association of Mn to beneficial ligands, e.g. organic acids. Cowpea cultivar TVu 1987 showed significantly lower NADH-*per*oxidase activity at even higher Mn concentrations in the apoplast. Since no significant differences in the occurrence and concentrations of organic acids in the apoplast between cvs TVu 91 and TVu 1987 have been observed (Maier, 1997), genotypic differences in Mn tolerance are probably not due to such differences.

*The particular role of phenols and non-enzyme components of the apoplast in NADH-*per*oxidase activity*

The nature of phenolic compounds had a significant effect on the functionality of PODs (Lee, 1977; Mäder and Füssl, 1982; Pedreño et al., 1987). This is comprehensible on the basis of their wide variance in structure and reaction behaviour (Rice-Evans et al., 1996). Already Kenten and Mann (1950) detected a significant influence of several phenolic compounds on the POD-catalysed oxidation of manganous pyrophosphate. Mn oxidation was stimulated by monohydroxy phenols, but not by dihydroxy and trihydroxy phenols. Phenols have been proposed to affect the peroxidase-catalysed NADH oxidation via different pathways. The stimulating effect of phenols on NADH oxidation was associated to an accelerated breakdown of compound III in the peroxidase-oxidase cycle (Yamazaki and Yokota, 1973; Halliwell, 1978) and to an enhanced NADH oxidation itself in the peroxidatic cycle (Mäder and Füssl, 1982; Pedreño et al., 1987). Additionally, an inhibitory effect on the formation of compound III was suggested (Mäder and Füssl, 1982). The time course of NADH oxidation showed a characteristic lag phase, followed by a high turnover rate of NADH (Yokota and Yamazaki, 1977; Pedreño et al., 1987). During the lag phase, formation of compound III from the ferric enzyme increased approximately exponentially (Yokota and Yamazaki, 1977). Furthermore, it was shown that trace amounts of H_2O_2 shortened the lag phase, and the addition of catalase delayed subsequent NADH oxidation (Yokota and Yamazaki, 1977; Pedreño et al., 1987). Therefore, the lag phase corresponds to the peroxidase-oxidase cycle (Pedreño et al., 1987). The length of the lag phase is significantly affected by phenols and influenced by the concentrations of enzyme and NADH (Yokota and Yamazaki, 1977). The additon of p-coumaric acid and coniferyl alcohol caused a lag phase, followed by a sudden and a rapid NADH oxidation. No distinct lag phase was observed in the presence of ferulic acid and guaiacol. The addition of sinapinic acid, alone or together with ferulic acid, inhibited NADH oxidation completely. Interestingly, the oxidation of NADH by purified proteins from the AWF showed a similar characteristic time course (Fig. 9). The NADH oxidation showed also an initial lag phase followed by rapid oxidation of NADH. Particularly the length of the initial lag phase was strongly affected by the origin of the AWF filtrate (Fig. 9, 10). The AWF-filtate from cv TVu 91 (Mn sensitive) shortened the lag phase and the AWF filtrate from cv TVu 1987 induced a delay of NADH oxidation (Fig. 9). Since no NADH oxidation was observed by AWF filtrates alone, particular the interaction between enzymes and apoplastic promoters

(cv TVu 91) or inhibitors (cv TVu 1987) governs the oxidation of NADH. The similar effect of phenolic compounds on the time courses of POD-catalysed IAA oxidation (Gelinas and Postlethwait, 1969; Lee, 1977) support the close relationship between phenolic compounds and the functionality of POD *per se*. Gelinas and Postlethwait (1969) reported a logarithmic increase in lag period by the addition of linearly increasing concentrations of boiled plant extracts from maize plants, containing ferulic acid. The similar effects of phenols and AWF filtrates on the functionality of NADH-*per*oxidase suggest that the phenol composition and concentration in the apoplast play a crucial role in Mn toxicity and particular Mn tolerance. The lower NADH-*per*oxidase activity in cv TVu 1987 at elevated levels of guaiacol-POD activity (Fig. 4) may be caused by the inhibitory effect of specific phenols on NADH oxidation present in the AWF of cv TVu 1987.

*Relationship between NADH-*per*oxidase and H_2O_2 formation and its role in Mn toxicity*

The presented results support the assumed linkage between NADH-*per*oxidase activity and H_2O_2 formation. The formation rate of H_2O_2 *in vitro* was significantly increased by Mn treatment in the Mn-sensitive cultivar (Fig. 5) and significantly correlated with NADH-*per*oxidase activity (Fig. 6). The addition of co-factors simultaneously influenced both NADH-*per*oxidase activities (Fig. 7) and H_2O_2 formation-rate (Fig. 8). Furthermore, H_2O_2 concentrations after addition of catalase were decreased (data not shown). As already discussed in relation to the functionality of NADH-*per*oxidase, H_2O_2 formation was also significantly affected by the addition of several phenols (Elstner and Heupel, 1976; Gross et al., 1977).

The development of Mn toxicity was attributed to an increased formation of H_2O_2 in the apoplast (Horst et al., 1999). Due to the close relationship of POD and NADH-*per*oxidase activity and the formation of H_2O_2 *in vivo*, a H_2O_2-producing and consuming POD system in the apoplast appears to be a potent mechanism for development and avoidance of Mn toxicity in the apoplast.

Effect of Mn treatment on phenol concentration and composition in the AWF and its role in Mn toxicity and tolerance

In relation to the presumably significant effect of phenolic compounds on the functionality of PODs, phenol concentration and phenol composition in the AWF of cowpea leaves were investigated.

In cowpea, phenol contents in the leaf tissue (water and NaOH extract, Maier 1997) and AWF of the Mn-sensitive cv TVu 91 were higher than in cv TVu 1987 (Fig. 11) or on the same level (data not shown). Therefore, a clearly positive correlation between Mn tolerance and the quantity of phenols neither in the tissue nor in the AWF is approvable, as proposed by Aoba (1986). The stimulating effect of Mn on PAL activity (Burnell, 1988; Engelsma, 1972) and phenol contents in the tissue (Langheinrich et al., 1992) support the hypothesis that Mn excess also induces the release of phenolic compounds into the leaf apoplast (Wissemeier and Horst, 1992). In the presented experiments, particular plants with marked expression of Mn toxicity symptoms showed an increase of phenol concentration in the AWF (Fig. 12). This is also reflected by the occurrence of additional peaks in the chromatogram of AWF from Mn-treated cv TVu 91 (Fig. 13B). Plants of cv TVu 91 and cv TVu 1987 with no or only light Mn toxicity symptoms showed no changes in apoplastic phenol concentrations. This indicates, that the release of phenolic compounds into the apoplast reflects rather a late stress response to excess Mn than a tolerance mechanism.

The differences of cowpea cultivars TVu 91 and TVu 1987 in phenol composition (Fig. 13A) might be causal for genotypic differences in Mn tolerance. As already mentioned, phenolic compounds are affecting the functionality of PODs. Minor genotypic deviations or modifications in phenol compositions might rule the formation of H_2O_2 and therefore the oxidation of Mn^{II} and phenolic compounds. This is indicated by the work of Pedreño et al. (1987, see above).

Detoxification of metals by sequestration with phenolic compounds was also dicussed (Heim et al., 2001, Kidd et al., 2001; Lavid et al., 2001). But the role of phenols in metal toxicity and tolerance *in vivo* is difficult to draw. This is due to the multitude of factors, affecting the behaviour of phenolic compounds in the plant system. Plant phenolic compounds show antioxidant and prooxidant activities as well, which depends on metal-reducing potential, chelator behaviour, pH, and solubility characteristics (Rice-Evans et al., 1996; Decker, 1997; Moran et al., 1997; Sakihama et al., 2002). Therefore, sequestration of

metals by phenolic compound in plants is not always supporting metal tolerance. Until now, the formation of brown-coloured Mn-phenol complexes in cowpea indicates always Mn sensitivity.

However, the identities of phenolic compounds detected in the AWF are unknown so far. Therefore, the behaviour of these phenols in the apoplast is not predictable.

CONCLUSION

The deposition of oxidized phenolic compounds and oxidized Mn in the cell wall of leaves of the sensitive cowpea cultivar TVu 91 is accompanied by an increase of POD activity and release of PODs and PR-like proteins into the leaf apoplast. The development of these indicators for Mn toxicity and therefore Mn sensitivity is probably accompanied by the formation of extracellular H_2O_2. Peroxidases were proposed to act as polyfunctional enzymes, thus catalysing the formation and consumption of H_2O_2. The catalytic activity of POD was divided into two main cycles, named peroxidase-oxidase and peroxidatic cycle. The functionality of POD as NADH-oxidase is significantly affected by the origin and concentration of substrates and co-factors. Phenolic compounds and Mn have an ambivalent effect on NADH-*per*oxidase activity. Water-soluble non-enzyme compounds of the AWF had similar effects on the time course of NADH oxidation as several phenolic compounds. Particular the differences in affecting NADH-*per*oxidase activity and the differences in apoplastic phenol composition of cowpea cultivars TVu 1987 (Mn tolerant) and TVu 91 (Mn sensitive) support the importance of apoplastic phenolic compounds on the functionality of NADH-*per*oxidase. Particular the inhibitory effect of AWF components from cv TVu 1987 on NADH-*per*oxidase is probably responsible for the higher tissue tolerance of this cultivar. Since NADH-*per*oxidase activity is not completely suppressed, Mn toxicity occurs also but significantly delayed.

We conclude from our results, that PODs in the AWF of cowpea are capable to produce and to consume H_2O_2 in the apoplast. For the H_2O_2-producing NADH oxidase cycle, the interaction between PODs and phenolic compounds in the leaf apoplast of cowpea is crucial for development and avoidance of Mn toxicity. The characterization of phenol composition in the apoplast will give new insights into the regulation of H_2O_2-producing PODs in the leaf apoplast at its importance for the mechanism of Mn toxicity and Mn tolerance in the leaves of cowpea.

CHAPTER 4:

DOES APOPLASTIC ASCORBIC ACID ENHANCE MANGANESE TOLERANCE OF *VIGNA UNGUICULATA* AND *PHASEOLUS VULGARIS*?

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(to be submitted)

ABSTRACT

In cowpea (*Vigna unguiculata*), the development of Mn toxicity is considered to be accompanied by the formation of reactive oxygen species, oxidized Mn and phenoxy radicals in the leaf apoplast. Ascorbic acid (AA) is a common antioxidant in plants and the oxidation of AA particular in the leaf apoplast contributes to the first line of defence against several biotic and abiotic stresses. A close relationship between ozone tolerance of plants and the capacity to maintain sufficient levels of AA in the leaf apoplast was postulated. The objective of the present study was to contribute to a better understanding of the role of AA in Mn leaf-tissue tolerance of cowpea and common bean (*Phaseolus vulgaris*). In agreement with previously shown results, the concentration of AA in the apoplastic washing fluid (AWF) and the ratio of AA/(AA+DHA) decreased within the first days of elevated Mn supply. After 3 days of Mn treatment, visible toxicity symptoms were clearly expressed and peroxidase activities were significantly increased, accompanied by a significant release of proteins into the apoplast. At this advanced stage of Mn toxicity, only 2% of reduced AA was measurable in the AWF. The ratio AA/(AA+DHA) in the leaf tissue was nearly unaffected and the total AA+DHA contents in the leaf tissue were even increased after prolonged Mn treatment and thus advanced expression of Mn toxicity. The application of AA solutions in the range of 4-10 µM via the petiole slightly enhanced Mn tolerance as indicated by the suppression of brown depositions and decreased peroxidase activities in the AWF. Common bean cultivars differing in ozone tolerance which has been reported to be due to a high AA availability in the leaf apoplast were studied for their Mn tolerance. Clear differences in Mn tolerance between the cultivars existed, however, these differences were not related to their ozone tolerance. From these results we conclude that the maintenance of sufficient AA levels in the leaf apoplast contribute to Mn tolerance, but does not fully explain genotypic differences in Mn tolerance in cowpea and common bean.

INTRODUCTION

Manganese (Mn) is a plant nutrient involved in various physiological processes (Campbell and Nable, 1988) as part of proteins and co-factor for a number of enzymatic reactions (Burnell, 1988). But under conditions of increasing Mn availability, e.g. in acid soils (Foy, 1984; Fox et al., 1991) and after steam sterilisation of substrates (Sonneveldt and Voogt, 1975), Mn concentrations in the plant tissue may lead to Mn toxicity characterized by the

formation of typical Mn toxicity symptoms and the reduction of plant growth and crop yield. Plant species (Andrew and Hegarty, 1969; Horiguchi, 1987; Hannam and Ohki, 1988; Singer and Havill, 1993) and cultivars within species (Nelson, 1983; Moroni et al., 1991; Sale et al., 1992; Mgema and Clark, 1995) show a high variability in resistance to Mn excess. A particular high genetic variability has been reported in the Mn-sensitive legumes soybean (*Glycine max.*) (Carter et al., 1975; Heenan and Carter, 1976), common bean (*Phaseolus vulgaris*) (Horst and Marschner, 1978) and cowpea (*Vigna unguiculata*) (Horst, 1980). First visible Mn toxicity symptoms in these species are brown spots on older leaves, followed by chlorosis, necrosis and leaf shedding (Horst, 1988). In cowpea, considerable genotypic differences in the expression of toxicity symptoms have been observed at comparable Mn leaf-tissue contents (Horst, 1980). Therefore, genotypic differences in response to high Mn supply are due to differences in leaf-tissue Mn tolerance.

Brown spots represent local accumulations of oxidized Mn and oxidized phenolic compounds in the cell wall (Horiguchi, 1987; Wissemeier and Horst, 1992). It has been hypothesised that the oxidation of Mn^{II} and phenolic compounds is catalysed by peroxidases in the leaf apoplast leading to the formation of Mn^{III} and phenoxy radicals (Kenten and Mann, 1950; 1956). We have recently shown that Mn excess leads to the enhanced release of peroxidases and further pathogenesis related like (PR-like) proteins into the leaf apoplast of cowpea (Fecht-Christoffers et al., 2003b). Peroxidases may not only be implicated in the oxidation of Mn and phenols but also in the formation of H_2O_2 (Elstner and Heupel, 1976; Gross et al., 1977). We presented evidence that Mn excess stimulates H_2O_2-producing PODs and H_2O_2-formation in the leaf apoplast of cowpea (Fecht-Christoffers et al., 2003c). This confirms our earlier results showing enhanced release of H_2O_2 from washed leaf segments from the Mn-sensitive cultivar TVu 91 precultured at toxic Mn supply (Horst et al., 1999). H_2O_2 is involved in the local triggering of pathogen-induced programmed cell death (PCD) during the hypersensitive response (HR) (Levine et al., 1994; Tenhaken et al., 1995; Desikan et al., 2001) and might induce PR-like proteins (Chamnongpol et al., 1998) which we also shown for Mn toxicity (see above). From these results we concluded that Mn-induced enhanced H_2O_2 production and thus oxidative stress in the leaf apoplast are key reactions in the expression of Mn toxicity (Fecht-Christoffers et al., 2003c).

Mn tolerance would then particularly depend on the capacity of the leaf tissue to prevent oxidative damage. In this regard antioxidants such as tocopherols, β-carotene, phenolic

compounds, and ascorbic acid (AA) may be important (Schmitz and Noga, 2000). The role of AA in stress resistance, growth, and cell signalling is well documented by several recent reviews (Polle and Rennenberg, 1993; Noctor and Foyer, 1998; Horemans et al., 2000; Smirnoff, 2000; Pignocchi and Foyer, 2003). The beneficial properties of (AA) for plant health was already exploited 40 years ago: spraying of AA-solutions on plants prevented plant damage by air-borne oxidizing agents (Freebairn and Taylor, 1960). Plants with high AA concentrations in the plant tissue proved to be more suitable to resist oxidative damage by ozone than plants with low AA concentrations (Lee et al., 1984; Lee, 1991). Particular cultivars of common bean maintaining higher AA concentrations in the leaf apoplast were less affected by ozone treatment (Burkey and Eason, 2002). Also, the higher stress sensitivity of the AA-deficient *Arabidopsis* mutant *soz1*, which accumulated only 30% of the normal ascorbate concentration, confirms a close relationship between the AA status of the plant tissue and its tolerance against environmental stresses (Conklin et al., 1996). Particular the concentration of reduced AA in the leaf apoplast plays a significant role in plant defence, because this compartment is strongly affected by abiotic and biotic stresses (Dietz, 1997; Vanacker et al., 1998b; Pignocchi and Foyer, 2003). Since the apoplast is considered to be the most important compartment for development and avoidance of Mn toxicity (see above), the maintenance of a high AA levels in the apoplast may also be important for Mn leaf-tissue tolerance. Indeed, Mn excess affected the level of AA in the leaf apoplast and in the leaf tissue (Horst et al., 1999; Fecht-Christoffers et al., 2003a). But an unequivocal prominent role of AA in the mechanisms of Mn tissue tolerance has not been clearly demonstrated.

The objective of the present study was to contribute to a better understanding of the role of AA in Mn leaf-tissue tolerance of cowpea and common bean using different experimental approaches.

MATERIAL AND METHODS

Plant material and cultivation

Plants of several cowpea (*Vigna unguiculata* (L.) Walp.) and common bean (*Phaseolus vulgaris* L.) cultivars (see figure legends) were grown hydroponically in a growth chamber under controlled environmental conditions (for experimental details see Fecht-Christoffers et al., 2003c). After preculture for at least 14 days, the $MnSO_4$ concentration in the nutrient solution was increased up to 20, 40, 50 and 100 µM $MnSO_4$ (for details see figure

legends). The duration of treatment for each experiment is given in figure legends. Control plants received 0.2 µM Mn continuously. All plants were harvested at the same day. The nutrient solution was changed two to three times a week to avoid nutrient depletion by more than 50%.

Ascorbic acid application

Ascorbic acid (AA) application experiments were carried out on plants of cowpea cultivar TVu 91. Freshly prepared AA solutions (4, 5 and 10 µM) with a pH adjusted to 5.8-6 with 1 M KOH were applied to the oldest full-expanded trifoliate leaves (leaf insertion I). Tissues soaked with AA solutions were wrapped around the petioles. Sleeves were covered with aluminium foil to prevent evaporation and light-induced destruction of AA. Treatment with AA solution was repeated every 24 h during the duration of the treatment. AA (leaf petiole) and elevated Mn (root) treatments were started simultaneously.

Quantification of Mn toxicity symptoms

Leaf discs (1.54 cm^2) were cut out at the base, middle and tip of the trifoliate leaf and incubated in EtOH for at least 3 days. Numbers of brown spots on decolourised leaf discs were counted. The density of brown spots per cm^2 leaf area was calculated.

Extraction of Apoplastic Washing Fluid (AWF)

Apoplastic washing fluid (AWF) was extracted by a vacuum infiltration/centrifugation technique. Leaves were cut from plants, weighted and infiltrated with demineralised water (dH_2O) by transferring the leaves into vacuum-resistant filtering flasks (DURAN®) filled with dH_2O. The pressure was reduced to 35 hPa (1 min) by using a water jet pump followed by slow relaxation for 2 min. Leaves were removed, dry blotted and were weighted again. AWF was recovered by centrifugation at 1324 *g* for 5 min at room temperature.

Extraction of AA and DHA from the leaf tissue

Approximately 0.15 g fresh leaf tissue was homogenized in 1 ml 2% meta-phosphoric acid/1 mM EDTA for 2 min at 4°C and 30^{s-1} (MM200, Retsch, Haan, Germany). After centrifugation (5000 *g*, 15 min, 4°C), the supernatant was used immediately for AA and DHA measurements.

Measurement of AA and DHA concentrations

AA and DHA concentrations were measured colorimetrically by direct measurement of reduced AA at λ=265 nm in a microplate reader (μQuant, BioTek Instruments GmbH, Neufahrn, Germany). Samples volumes of 200 µl AWF or 50 µl supernatant of the leaf extract, respectively, were mixed with 100 mM sodium-phosphate buffer (pH 6.8) to give a final volume of 300 µl. Absorptions were measured with addition of (a) 1 mM dithiothreitol (DTT), (b) 0.1 unit ascorbate oxidase (AAO) or (c) sodium-phosphate buffer. AA and DHA concentrations were calculated as follows: AA (µM) = Abs_c-Abs_b; DHA (µM)=Abs_a-Abs_c. Calibration curves were linear from 0 to 5 µM (for AWF samples) and from 0-400 µM (for leaf tissue samples), respectively.

Measurement of guaiacol-peroxidase (POD) activity

Guaiacol-POD activities in the AWF were determined spectrophotometrically at λ=470 nm (UVIKON 943, BioTek Instruments GmbH, Neufahrn, Germany) following the H_2O_2-depending oxidation of guaiacol. Samples were mixed with guaiacol solution [20 mM guaiacol in 10 mM sodium-phosphate buffer (pH 6) and 0.03% (w/w) H_2O_2]. Enzyme activities were calculated using the molar extinction coefficient ε= 26.6 $mM^{-1}cm^{-1}$.

Measurement of catalase activity

The catalase activity in the AWF was measured following the decrease in absorbance of H_2O_2 at λ=240 nm. The assay was made up of 50 mM potassium-phosphate buffer (pH 7) and 30 mM H_2O_2 (ϵ=0.036 $mM^{-1}cm^{-1}$).

Mineral analysis

For the detection of Mn in the bulk-leaf tissue, the central leaf veins of the middle leaflets of trifoliate leaves were discarded and 0.5 – 1 g leaf tissue was dried at 65°C. Drying was followed by dry ashing (480°C, 8h) and dissolving the ash in 6 M HCl with 1.5% (w/v) hydroxylammonium chloride and dilution (1:10) with ddH_2O. AWF was diluted 1:10, whereas HCl and hydroxylammonium chloride was added to give a final concentration of 0.6 M HCl and 0.15% (w/v) hydroxylammoniumchloride. Measurements were carried out by optical emission spectrometry, inductively coupled plasma (Spektro Flame, Spectro, Kleve, Germany).

Statistical analysis

The number of sample replications is given in figure legends. Each sample was collected from leaves (leaf insertion I or II, see figure legends) of two plants. Regression analysis, analysis of variance, and multiple comparisons of means (Tukey test) were carried out using SAS 8e (SAS Institute Inc., Cary, North Carolina, USA). Levels of significance in graphs are given by $^{+}$, *, **, *** for $p<0.1$, 0.05, 0.01, 0.001. Different letters are significantly different at $p<0.05$ (Tukey).

RESULTS

Response of cowpea cultivars to increased Mn supply

The cowpea cultivars Solojo, TVu 91, TVu 1977, TVu 1987 and TVu 354 showed significant differences in Mn leaf tissue contents. However, the differences in the expression of Mn toxicity symptoms (brown spots) particularly in the range from 0.5 to 1.25 µmol Mn per g fresh weight cannot be explained by these differences (Fig. 1).

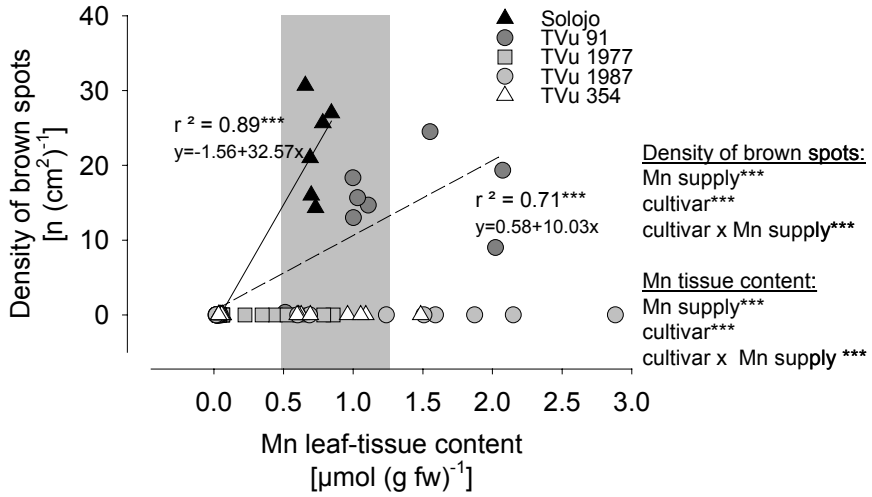

Figure 1 Relationships between Mn tissue contents and the density of brown spots of five cowpea cultivars. Plants were grown in nutrient solution with 50 µM $MnSO_4$ for 4 days, whereas control plants received 0.2 µM Mn continuously. The second oldest full-expanded trifoliate leaf (leaf insertion II) was used for analysis. n=8. Results of the analysis of variance are given according to their level of significance as ***, **, * for $p<0.001$, 0.01, 0.05, respectively.

Cv Solojo showed the highest density of brown spots, followed by cv TVu 91. These cultivars can be classified as Mn sensitive. The cultivars TVu 1977, TVu 1987 and

TVu 354 did not show visible toxicity symptoms and can be rated as Mn tolerant. The appearance of brown spots in cvs Solojo and TVu 91 was accompanied by a higher activity of peroxidases (PODs) in the leaf apoplastic washing fluid (AWF) (Fig. 2A).

In these cultivars, activity of apoplastic PODs at optimum low Mn supply was even higher than at high Mn treatments of the other cultivars. The increased Mn supply also caused a slight activation of catalase in the AWF over all cultivars (Fig. 2B). Mn-tolerant cv TVu 1987 was characterised by a particularly high catalase (CAT) activity at both Mn supplies. However, the other cultivars did not show a pattern of catalase activity which could be related to the genotypic differences in Mn tolerance.

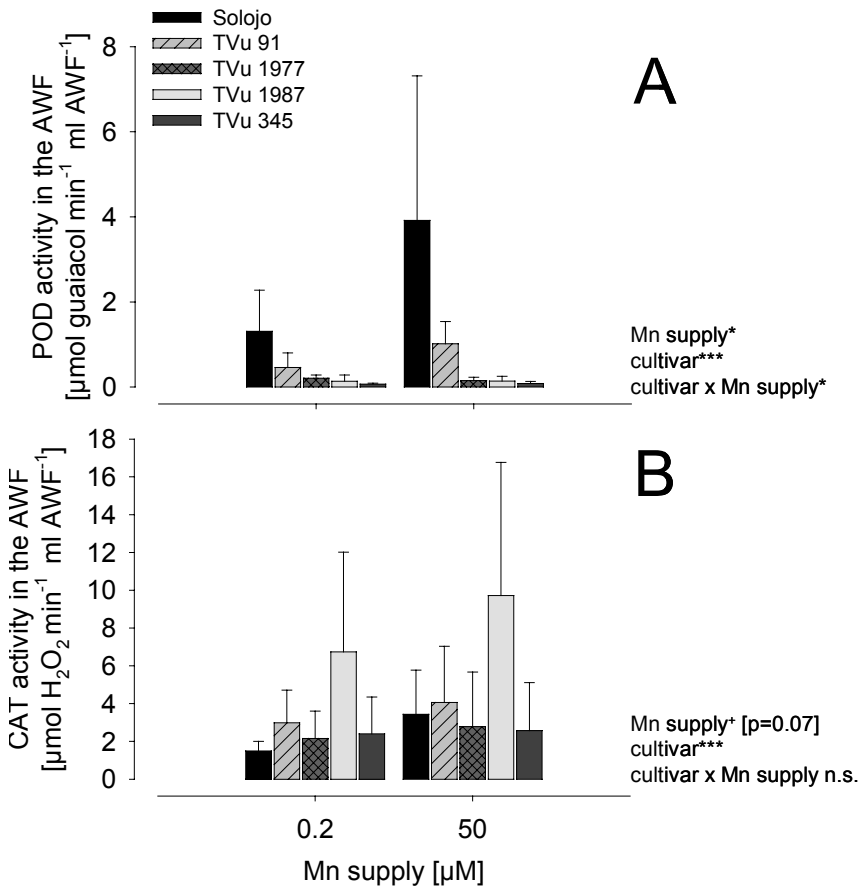

Figure 2 Effect of increased Mn supply on the activity of (A) peroxidase (POD) and (B) catalase (CAT) in the leaf AWF of five cowpea cultivars. Plants were grown in nutrient solution with 50 µM $MnSO_4$, whereas control plants received 0.2 µM Mn continuously. The second oldest full-expanded trifoliate leaf (leaf insertion II) was used for analysis. n=8. Results of the analysis of variance are given according to their level of significance as ***, **, * or + for p<0.001, 0.01, 0.05 and 0.1, respectively.

The development of Mn toxicity in cv TVu 91 during 3 days of Mn treatment

With increasing duration of treatment, Mn was readily taken up by the plants and transported to the leaves. The Mn leaf-tissue content significantly increased from the 2nd day of treatment. This was accompanied by a significant expression of visible Mn toxicity symptoms and enhanced activity of PODs in the leaf AWF (Table 1).

Table 1 Effect of increased Mn supply for a treatment period of 3 days on the Mn tissue content, formation of brown spots, apoplastic peroxidase (POD) activity and protein concentration in the AWF. Plants of cowpea cultivar TVu 91 were grown in nutrient solution with 50 µM $MnSO_4$, whereas control plants received 0.2 µM Mn continuously. The first trifoliate full-expanded leaf (leaf insertion I) was used for analysis. n=9. Mean values are significantly different at $p<0.05$ (Tukey test) as given by different letters.

Duration of treatment [d]	Mn leaf tissue content [µmol $(g\ fw)^{-1}$]		Density of brown spots [n $(cm^2)^{-1}$]		POD activity [µmol guaiacol min^{-1} ml AWF^{-1}]		Protein concentration in the AWF [µg ml^{-1}]	
0	0.2239	a	0.0148	a	1.1907	a	27.4424	a
1	0.2554	a	0.1082	a	1.3707	a	35.9106	a
2	0.8184	b	26.3889	b	2.8498	b	44.3448	a
3	0.8867	b	59.4877	c	4.8334	c	88.6509	b

A significant increase in apoplastic protein concentrations in the AWF was measured from the 3rd day of Mn treatment. The concentration of reduced ascorbic acid (AA) decreased in the AWF with duration of the Mn treatment (Fig. 3). While after the 1st day of Mn treatments only a tendency of decreased AA concentrations in the AWF could be observed, the concentrations of oxidized ascorbate (dehydroascorbate, DHA) in the AWF and leaf tissue increased significantly. With prolonged duration of the Mn treatment, no further increase in DHA concentrations was detected. However, the AA concentrations in the AWF decreased steadily to a very low level after 3 days of Mn treatment, whereas the AA contents in the leaf tissue remained rather stable. After 2 days of Mn treatment even a significantly higher AA content was measured.

The total ascorbate concentrations in the AWF remained stable over the Mn treatment duration, whereas the leaf tissue-contents significantly increased after 2 days of Mn treatment (Fig. 4). Over the 3 days of Mn treatment period the ratio of reduced AA to total ascorbate (AA+DHA) was unaffected in the leaf tissue. However, the high ratio of reduced AA in the AWF of control plants decreased within one day of Mn treatment significantly. Only 2% of AA remained in the AWF after 3 days of Mn treatment. This picture did not change even after longer Mn treatment (not shown).

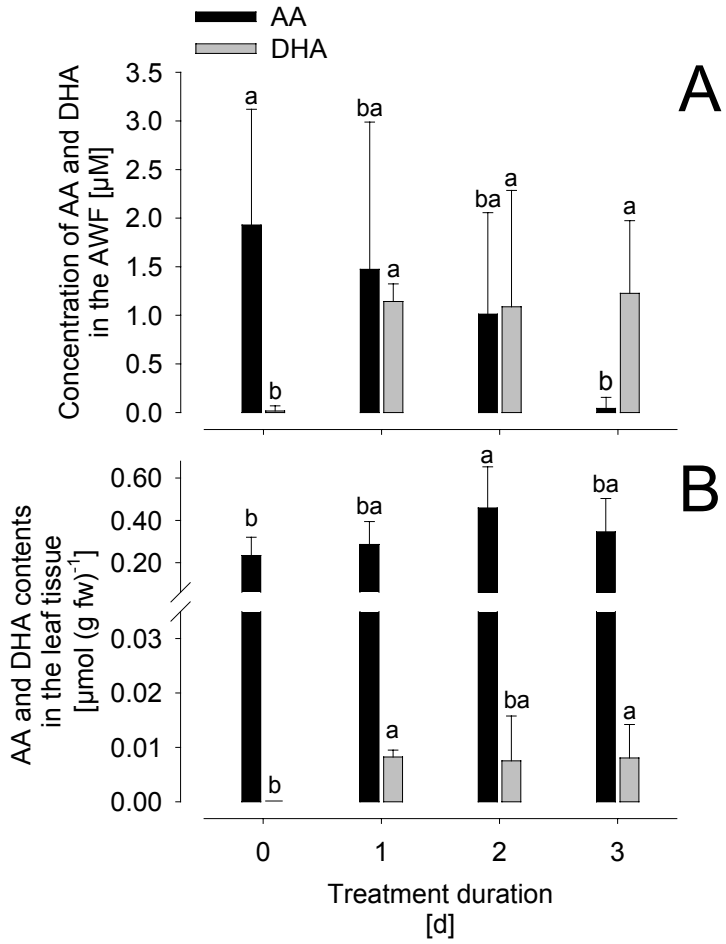

Figure 3 Effect of increased Mn supply for a treatment period of 3 days on (A) the concentration of AA and DHA in the AWF and (B) on the AA and DHA content in the leaf tissue. Plants of cowpea cultivar TVu 91 were grown in nutrient solution with 50 µM $MnSO_4$ for 4 days, whereas control plants received 0.2 µM Mn, continuously. The first trifoliate full-expanded leaf (leaf insertion I) was used for analysis. n=9. Mean values are significantly different at $p<0.05$ (Tukey test) as given by different letters.

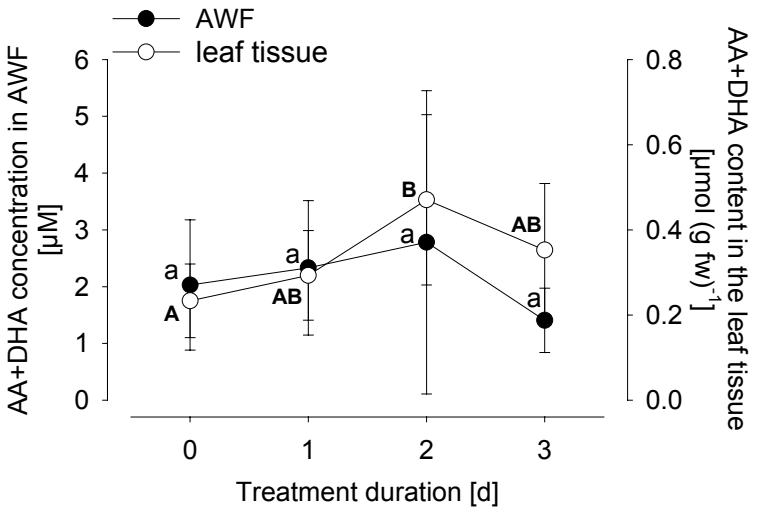

Figure 4 Effect of increased Mn supply for a treatment period of 3 days on the total ascorbate AA+DHA in the AWF and in the leaf tissue. Plants of cowpea cultivar TVu 91 were grown in nutrient solution with a Mn concentration of 50 µM $MnSO_4$ for 4 days, whereas control plants received 0.2 µM Mn continuously. The first trifoliate full-expanded leaf (leaf insertion I) was used for analysis. n=9. Mean values are significantly different at $p<0.05$ (Tukey test) as given by different letters (small letter=AWF; capital letters=leaf tissue).

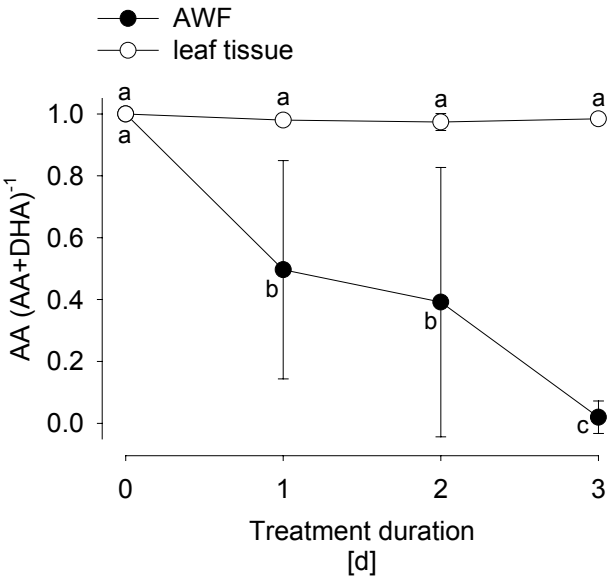

Figure 5 Effect of increased Mn supply for a treatment period of 3 days on the ratio of reduced ascorbic acid (AA) to total ascorbate (AA+DHA) in the AWF and in the leaf tissue. Plants of cowpea cultivar TVu 91 were grown in nutrient solution with 50 µM $MnSO_4$, whereas control plants received 0.2 µM Mn continuously. The first trifoliate full-expanded leaf (leaf insertion I) was used for analysis. n=9. Mean values are significantly different at $p<0.05$ (Tukey test) as given by different letters.

The effect of ascorbic acid application on the development of Mn toxicity

Application of 4 µM AA did not yield significant reductions of Mn toxicity symptoms and POD activities in the leaf AWF although there was a tendency of a reduced number of brown spots and POD activity by 4 µM AA application (Fig. 6). It has to be mentioned that the expression of Mn toxicity in this experiment was rather week as shown by the small number of brown spots and low stimulation of POD activity.

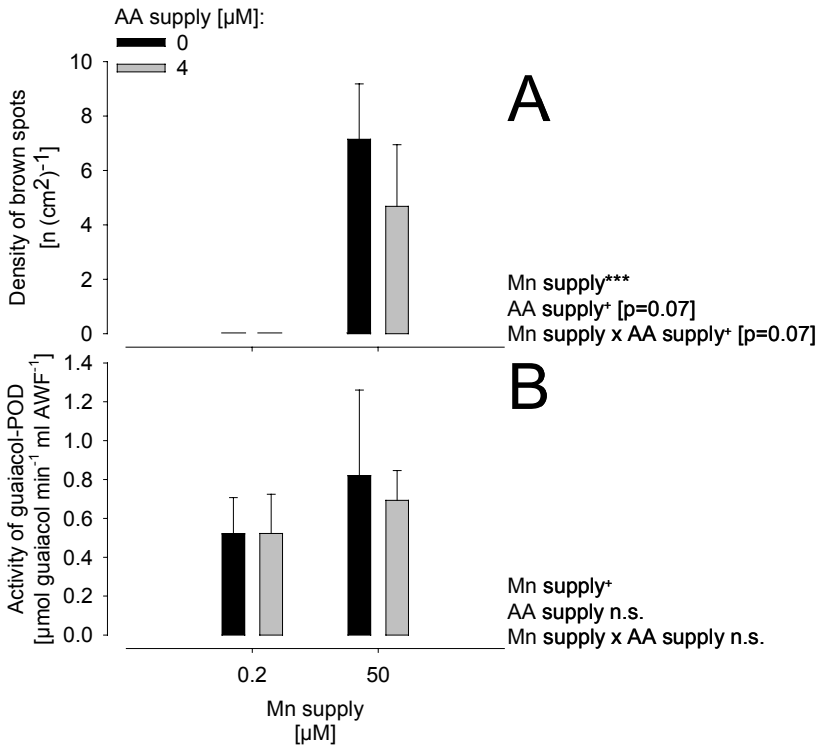

Figure 6 Effect of ascorbic acid application via the petiole on (A) the expression of brown spots and (B) the activity of apoplastic PODs in the leaf AWF. Plants of cowpea cultivar TVu 91 were treated with 50 µM Mn for 4 days, whereas control plants received 0.2 µM Mn continuously. The first trifoliate full-expanded leaf (leaf insertion I) was used for analysis. n=6. Results of the analysis of variance are given according to their level of significance as ***, **, * or + for p<0.001, 0.01, 0.05 and 0.1, respectively.

The application of 5 µM ascorbic acid (AA) solution to the leaves *via* the petiole reduced the expression of visible Mn toxicity symptoms significantly (Fig. 7A).

The effect of 10 µM AA was less and non-significant. However, the activity of Mn treatment-enhanced POD activity was highly significantly reduced by both AA treatments (Fig. 7B). Since it could not be excluded that the AA concentrations applied were already in the supraoptimal range a set of experiments with lower AA concentrations was conducted.

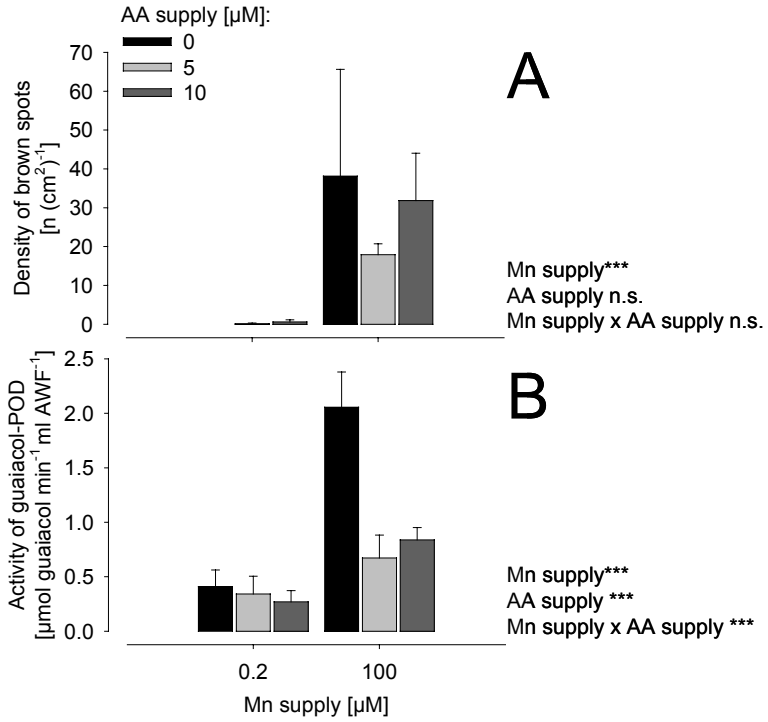

Figure 7 Effect of ascorbic acid application via the petiole on (A) the expression of brown spots and (B) the activity of apoplastic PODs in the leaf AWF. Plants of cowpea cultivar TVu 91 were treated with 100 µM $MnSO_4$ for 4 days, whereas control plants received 0.2 µM Mn continuously. The first trifoliate full-expanded leaf (leaf insertion I) was used for analysis. n=4. Results of the analysis of variance are given according to their level of significance as ***, **, * for $p<0.001$, 0.01, 0.05, respectively.

Mn toxicity in cultivars of Phaseolus vulgaris, differing in ozone tolerance

The ozone tolerance of *Phaseolus vulgaris* cultivars has been related to their capacity to maintain elevated AA concentration in the apoplast (see above). Since information about ozone tolerance of cowpea cultivars were not available *Phaseolus vulgaris* cultivars differing in ozone tolerance were studied regarding their Mn tolerance.

In comparison to cowpea, plants of common bean showed similar Mn contents in the leaf tissue after treatment with 20 and 40 µM $MnSO_4$ during the last 4 days of cultivation (Fig. 8). In contrast, the Mn concentrations in the AWF were up to 4 times higher in common bean than in cowpea (for comparison see Fecht-Christoffers et al., 2003b). A significant relationship between Mn tissue contents, the density of brown spots (Fig. 9) and the activity of apoplastic POD (Fig. 10) was given for all tested common bean cultivars. Based on the expression of brown spots, cvs Provider and S 144 showed highest density of brown spots, followed by cvs S 156 and Tenderette. However, the significant differences in expression of symptoms were marginal. The cultivars showed significant different levels of

POD activities, but the Mn induced enzyme activation was similar. Finally, no significant differences in Mn sensitivity and tolerance of the snap bean cultivars Provider, Tenderette, S 144 and S 156 (in order of increasing ozone sensitivity) were found.

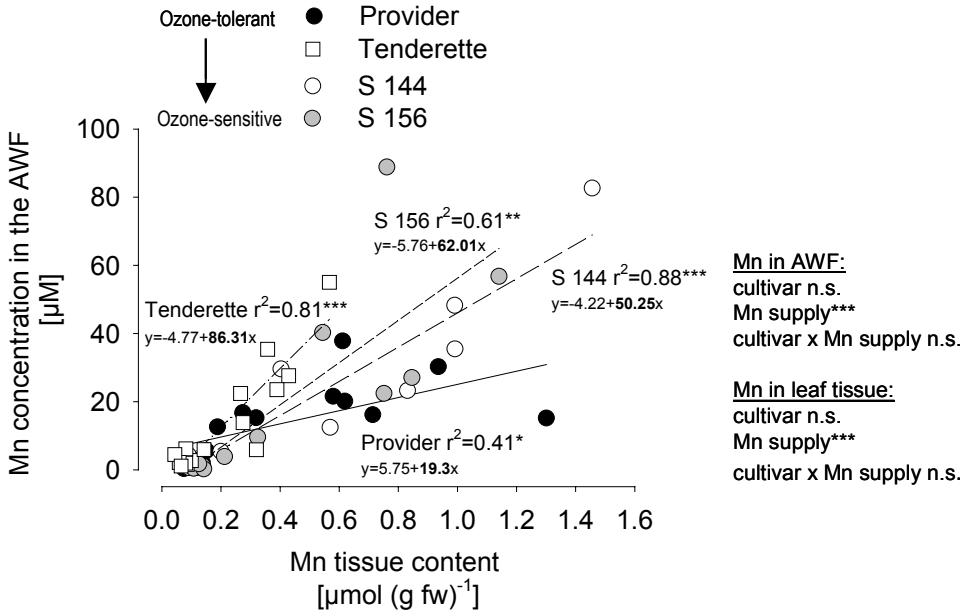

Figure 8 Relationship between the Mn tissue content and the Mn concentration in the AWF of four common bean cultivars differing in ozone tolerance. Plants were treated with 20 and 40 µM $MnSO_4$ for 5 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * for p<0.001, 0.01, 0.05, respectively.

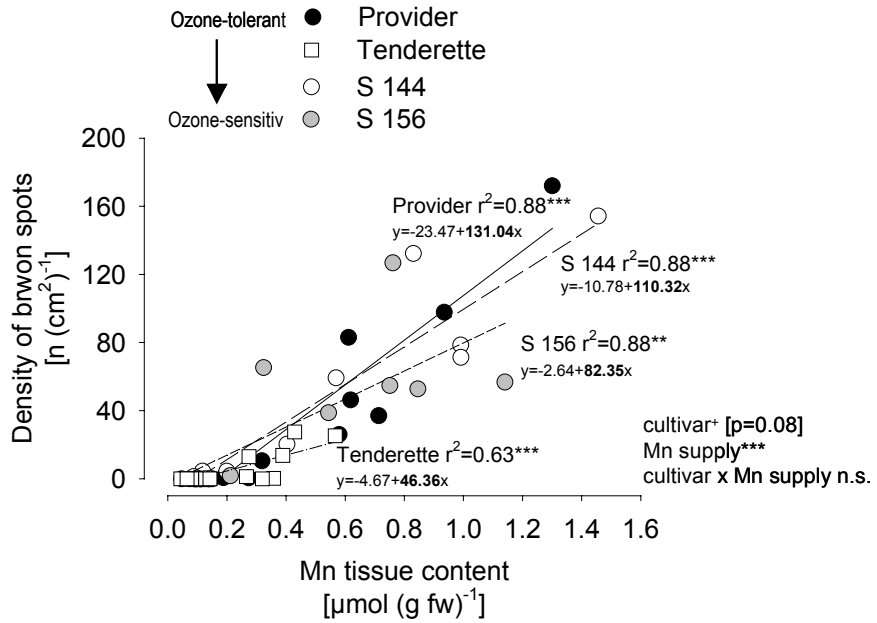

Figure 9 Relationship between the Mn tissue content and the density of brown spots in the leaf AWF of common bean cultivars differing in ozone tolerance. Plants were treated with 20 and 40 µM $MnSO_4$ for 5 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * , + for p<0.001, 0.01, 0.05, 0.1, respectively.

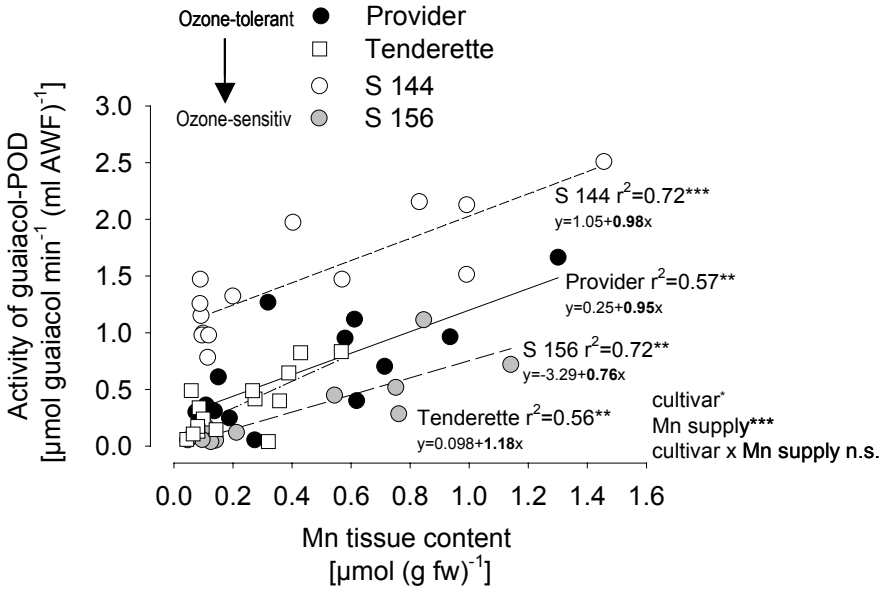

Figure 10 Relationship between the Mn tissue content and the activity of guaiacol-POD in the leaf AWF of common bean cultivars differing in ozone tolerance. Plants were treated with 20 and 40 µM $MnSO_4$ for 5 days, whereas control plants received 0.2 µM Mn continuously. Results of the analysis of variance are given according to their level of significance as ***, **, * for p<0.001, 0.01, 0.05, respectively.

DISCUSSION

The response of five cowpea cultivars to increased Mn supply

The presented results here confirm the previously shown close relationship between the expression of Mn toxicity symptoms (brown spots) and the activity of peroxidases in the leaf apoplast of cowpea (Fecht-Christoffers, 2003a, b, c). The activity of free movable PODs in the AWF proved to be a reliable indicator for Mn toxicity. The cowpea cultivars Solojo and TVu 91 can be classified as Mn sensitive. Beside peroxidase, catalase is the second major enzymatic system removing hydrogen peroxide in plants (Willekens et al., 1995). Apoplastic catalase was sometimes activated by pathogen infection (Vanacker et al., 1998a; Patykowski and Urbanek, 2003), but a decrease of catalase activity after inoculation was also reported (Vanacker et al., 1998b). Also, Cd treatment has been reported to increase (Metwally et al., 2003) or decrease catalase activity (Schützendübel et al., 2001). Schützendübel et al. (2001) postulated a loss of Cd-induced "antioxidative capacity" due to a depletion of glutathione (GSH) and inhibition of antioxidative enzymes, which probably causes H_2O_2 accumulation. Particular the inhibition of the thiol-containing glutathione-reductase (GR) has been ascribed to the affinity of Cd for sulphides. A Mn-induced decrease of antioxidative enzyme activity was observed in cotton (Sirkar and Amin, 1974). However, although Mn may also bind to sulphides, the affinity is much less than that of Cd (Nieboer and Richardson, 1980). Our own data did not suggest an inhibitory effect of Mn on GR (Fecht-Christoffers et al., 2003a) and catalase (Fig. 2). In common bean, thiol-containing compounds, ascorbate peroxidase and glutathione reductase were also not decreased by Mn treatment (González et al., 1998). Therefore, the proposed increase of apoplastic H_2O_2 is probably not related to an inhibition of catalase. Since only data from long-term experiments are available, detection of catalase activities and H_2O_2 concentrations in the leaf apoplast during initial stages of Mn toxicity are necessary to clarify the role of catalase in Mn toxicity.

The effect of Mn excess on the AA pool in the leaf apoplast and the entire leaf tissue of the Mn-sensitive cowpea cultivar TVu 91

Ascorbic acid is the most abundant antioxidant in the leaf tissue. Once synthesized, AA is transported to different compartments within the plant tissue (Wheeler et al., 1998; Siendondes et al., 1999). Although the plant apoplast contains only 5-10% of the total AA

(Horemans et al., 2000), this low molecular weight antioxidant contributes to the first defence line together with antioxidative enzymes of the leaf apoplast (Vanacker et al., 1998b; Dietz, 1997). Particular investigations on ozone toxicity showed the involvement of apoplastic AA in ozone-induced defence processes (Castillo and Greppin, 1988; Luwe et al., 1993). The ascorbic acid-deficient Arabidopsis mutant *soz1* showed increased sensitivity to ozone, sulfur dioxide, and ultraviolet B irradiation, which was partly reversed by treatment with AA and L-galactono-1,4-lactone, a precursor of AA (Conklin et al., 1996). Transgenic tobacco plants over-expressing cucumber ascorbate oxidase showed also increased sensitivity to ozone exposure (Sanmartin et al., 2003).

Similar to the effect of ozone on the AA status in the inter- and intracellular space of leaf tissues (Luwe et al., 1993), Mn excess also affected the AA pool in the leaf tissue of cowpea. The Mn-induced effects reported here showed analogies to the documented ozone effects. For instance, the redox state of AA is stronger affected by ozone and Mn in the apoplast than intracellularly (Luwe et al., 1993). Since the leaf apoplast is probably the primary site of ozone and Mn perception, the AA level of this compartment is affected first. Oxidized ascorbate from the apoplast is regenerated directly in the apoplast or transported from the apoplast into the cytoplasm (see review Smirnoff, 2000). On the first day of Mn treatment, increased AA turnover is indicated by a significant increase of DHA concentrations in the leaf apoplast and DHA contents in the leaf tissue (Fig. 3). The AA/(AA+DHA) ratio is significantly decreased as early as one day after the beginning of the Mn treatment (Fig. 5). Since no significant change in AA levels was observed at this stage of Mn toxicity (Fig. 3), the regeneration systems maintained a relatively high reduction capacity in the leaf apoplast. This is in line with a low expression of toxicity symptoms (Tab. 1) indicating a moderate stage of Mn toxicity. A further decline of AA concentration and AA ratio in the apoplast was observed with ongoing duration of Mn treatment until the AA ratio reached a level as low as approximately 2% suggesting the transition from a reduced to an oxidized state of the leaf apoplast. This is also indicated by the significant increase of apoplastic POD activities (Tab. 1). The inability to maintain high AA levels in the apoplast might be due to a restricted regeneration capacity and/or transport from the symplast (Rautenkranz et al., 1994; Horemans et al., 1997). The availability of AA in the leaf apoplast is probably mainly dependent of the developmental stage of the leaf. Since AA inhibits cross-linking reactions in the apoplast (Takahama and Oniki, 1992; Takahama, 1993), a high availability of reduced AA in young tissues is reported and probably required for unhindered growth (Sánchez et al., 1997) and enhanced

ozone tolerance of younger leaves (Lee et al., 1984; Luwe et al., 1993). Therefore, older tissues are *per se* probably more restricted in providing sufficient AA levels in the apoplast than young tissues. This might also partially be the cause for significant differences in Mn tolerance of old and younger leaves of cowpea: older leaves with low AA levels in the AWF are Mn sensitive and younger leaves with high AA concentrations in the AWF are Mn tolerant (Horst et al., 1999). The significant Mn-induced increase of total ascorbate in the leaf tissue (Fig. 4) indicates a stimulation of AA formation. Particular ozone-tolerant cultivars showed such an adaptation to ozone exposure (Lee et al., 1994). In cowpea, the AA/(AA+DHA) ratio in the cytoplasm remained at a high level even after 3 days of Mn treatment. In contrast, we reported also a complete oxidation of AA not only in the apoplast but also in the symplast. In the symplast, at least 45% of the total AA is located in cytoplasmic organelles e.g. in the vacuole (20-30%) and in chloroplasts (25-30%) (Horemans et al., 2000). A Mn-induced complete oxidation of cytoplasmic AA represents a deep impact on the physiology and compartmentalisation of the leaf tissue.

Ascorbic acid is a substrate for ascorbate peroxidase (EC 1.11.1.11) and class III peroxidases (EC 1.11.1.7) (Mehlhorn et al., 1996). Castillo and Greppin (1986) documented a higher affinity of cationic (alkaline) than anionic (acidic) PODs to AA. Since cationic PODs were proposed to respond more rapidly to stress than anionic PODs (Gaspar et al., 1985) an early ozone-induced consumption of AA might be caused by a stimulated oxidation of AA by PODs in the apoplast. But in cowpea, Mn-induced increase of AA oxidation by enzymes in the AWF was not detected (data not shown). Therefore, an AA oxidation by free movable PODs in the apoplast is improbable. Ascorbic acid may control the phenol oxidation by peroxidases in the leaf apoplast (Takahama and Oniki, 1992; Takahama, 1993) acting as the secondary electron donator leading to phenoxyradicals and thus an inhibition of the radical chain reaction. Since the POD-catalysed oxidation of phenolic compounds and Mn^{II} was considered to be the key reaction in Mn toxicity (Horst et al., 1999), ascorbate might interrupt the oxidation of phenolic compounds and Mn^{III} by scavenging phenoxy radicals. Additionally, the activity of H_2O_2-producing NADH-*per*oxidase in the AWF of needles of Norway spruce was also strongly affected by AA *in vitro*. The presence of AA caused a delay of NADH oxidation (Otter and Polle, 1994) indicating an interference with the oxidative cycle of peroxidase. Since in this cycle the intermediate $O_2^{\cdot -}$ is formed, AA might scavenge this precursor of H_2O_2 irreversibly. Therefore, AA is probably a potential candidate to control cross-link reaction in the apoplast by affecting the production of H_2O_2. In relation to Mn toxicity, NADH-

*per*oxidase is significantly induced by Mn supply (Fecht-Christoffers et al., 2003b,c). The Mn-induced increase of AA oxidation in the leaf apoplast might, therefore, represent a consequence of a Mn-enhanced NADH-*per*oxidase activity. The aggravation of Mn toxicity in spite of the decrease of the AA ratio in the apoplast indicate that the efficiency of this antioxidant system preventing Mn-induced oxidative damage is limited. In addition to the function of AA as a scavenger for oxidative compounds, changes in AA levels in the apoplast were also considered to act as a signalling system (Pignocchi and Foyer, 2003). Interestingly, leaves of the Arabidopsis mutant *vtc1* showed an induction of PR-like proteins, e.g. β-1,3 glucanase, chitinase, thaumatin-like proteins, and pathogenesis-related proteins (Pastori et al., 2003). In cowpea, Mn excess induced the expression and release into the leaf apoplast of PR-like proteins, which were comparable to those found by Pastori et al. (2003) (Fecht-Christoffers et al., 2003b). Until now, the signalling pathway of Mn-induced expression of PR-like proteins is unknown. But the significant decrease of AA-ratio in the apoplast of cowpea might potentially induce these stress-related proteins. The suppression of PR-like proteins by feeding of the *vtc1* mutants with AA confirms the impact of AA on the regulation of the genes coding the proteins.

The effect of AA application on Mn toxicity

The work of Pastori et al. (2003) demonstrates the beneficial effect of AA application on the suppression of PR-like proteins in AA-deficient *Arabidopsis* mutants. The beneficial effect of AA application to plants was already exploited 40 years ago (Freebairn and Taylor, 1960). In general, the application of antioxidants on plants is used to prevent tissue damage by UV irradiation (Schmitz-Eibinger and Noga, 2001). However, the excessive AA treatment of leaves caused damages of the leaf tissue (Freebairn and Taylor, 1960). At the beginning of our AA-treatment experiments with cowpea, we also observed leaf damage after application of 1 and 0.1 mM AA solutions (data not shown). Beneficial effect of AA on Mn toxicity were only demonstrated by the application of 5 and 10 µM AA (Fig. 7). Also the application of 4 µM AA caused a slight suppression of Mn toxicity symptoms and POD activity (Fig. 6). Already in the control plants, a decrease of POD activity was observed (Fig. 7). Since control plants of Mn-sensitive cowpea cultivars showed higher POD activities than controls of Mn-tolerant cultivars (Fig. 2A), the Mn concentration in the nutrient solution during preculture might induce higher leaf apoplastic POD activities in Mn-sensitive cultivars. In Mn-treated plants, especially the application of 5 and 10 µM

AA induced a significant decrease of POD activity. However, brown depositions were not statistically significantly reduced. This indicates that brown depositions are not necessarily formed by released POD. Cell wall-bound peroxidases are also activated by Mn treatment (Fecht-Christoffers et al., 2003a). Therefore, they are potential candidates to catalyse the Mn-induced browning of the tissue. AA did not suppress phenol oxidation by cell wall-bound PODs completely (Takahama, 1993; Otter and Polle, 1994). Provided that cell wall-bound PODs are involved in the browning of leaf tissue, the application of AA had probably minor effects on the suppression of brown depositions. However, the application of 4, 5 and 10 µM presumably prevented the release of POD into the leaf apoplast. This might be due to an increased availability of reduced AA in the leaf tissue with accompanied delay of the induction of stress-related proteins (see above). In the leaf apoplast, no significant changes of AA and DHA levels by AA application were observed (data not shown). It is already known that feeding of plants with AA does not necessarily increase the total AA pool in plant tissues (Mozafar and Oertli, 1993). Pallanca and Smirnoff (2000) showed that exogenous AA caused a decrease of AA synthesis from ^{14}C-glucose decreased within 3h of AA feeding. Furthermore, the rate of AA synthesis decreased and the rate of AA turnover increased linear with increasing AA pools size. However, provided that AA application controls the AA synthesis and AA turnover in plants by a direct “feedback”, the sense of AA application as a preventive measure is questionable.

The Mn tolerance of common bean cultivars differing in ozone tolerance and AA contents in the leaf apoplast

The selection and breeding of cultivars with a high antioxidative capacity could be a more promising strategy than the leaf application of AA. The overexpression of genes coding for antioxidative enzymes and the synthesis of antioxidative compounds *in vivo* by genetic transformation has not always resulted in the desired enhanced stress tolerance (Foyer et al., 1994; Rennenberg and Polle, 1994; Foyer et al., 1995; Noctor et al., 1998; Strohm et al., 1999). However, plants with high AA concentrations in the plant tissue were shown to avoid oxidative damage by ozone more successful than plants with low AA concentrations (Lee et al., 1984; Lee, 1991). The ozone tolerance of two common bean cultivars was associated to elevated AA contents in the apoplast (Burkey and Eason, 2002). But investigations with seven common bean cultivars differing in ozone tolerance gave

conflicting results, which were attributed to particular experimental conditions (Burkey and Eason, 2002; Burkey et al., 2003). However, the common bean cultivars Provider and Tenderette showed consistently high AA contents in the tissue and apoplast and were classified as ozone-tolerant, whereas the cultivars S 144 and S 156 were strongly affected by ozone fumigation and cv S 156 had low AA contents in the apoplast. In our studies, the cvs Provider and Tenderette showed significant differences in the expression of Mn toxicity symptoms (Fig. 9). Cv. Provider is classified as ozone-tolerant, but was strongly affected by excess Mn. Therefore, a simple relationship between a high availability of AA in the apoplast or leaf tissue and the ability to avoid Mn toxicity is unlikely which confirms our previously drawn conclusions (Fecht-Christoffers, 2003a). Since we applied a rather severe Mn toxicity stress to the plants, we cannot exclude that elevated levels of AA particular in the leaf apoplast might confer tolerance to low levels of oxidative stress induced by Mn. However, since major differences in Mn tolerance expressed in spite of the high stress level we conclude that the maintenance of higher AA level in the apoplast may contribute to, but are not decisive for genotypic differences in Mn tolerance in common bean as well as in cowpea

CONCLUSION

The measurement of peroxidase activity in the leaf apoplast is a reliable parameter for the characterization of Mn toxicity in cowpea and common bean. The enhanced POD and catalase activities are probably caused by Mn-induced formation of H_2O_2 in the leaf apoplast. Peroxidases were proposed to be the key enzyme in Mn toxicity. During catalyses of H_2O_2-formation and H_2O_2-consumption reactive intermediates are produced. Ascorbate is an effective scavenger in the apoplast. The Mn induced production of reactive compounds in the leaf apoplast is indicated by a strong decline of AA in the leaf apoplast. The suppression of Mn toxicity symptoms and peroxidase activity by AA application confirm the hypothesis, that the redox state of the apoplast is dramatically affected by excess Mn. AA in the leaf apoplast might act in the first line of defence, but did not prevent Mn-induced leaf injury at high stress level. The signalling pathway at the beginning of Mn toxicity is unknown so far. But the Mn-induced decline of AA in the apoplast might serve as a signal from the apoplast to the symplast.

CHAPTER 5:

EARLY EVENTS IN THE LEAF APOPLAST OF COWPEA (*VIGNA UNGUICULATA*) INDUCED BY MN EXCESS

Marion M. Fecht-Christoffers and Walter J. Horst
(to be submitted)

ABSTRACT

The formation of brown spots is the first visible indicator of Mn toxicity. H_2O_2-consuming peroxidases (PODs) were considered to catalyse the formation of MnO_2 and oxidized polyphenols appearing as brown depositions in the cell wall. Since most investigations were carried out with plants showing visible Mn toxicity symptoms, the presented work here focusses on early physiological changes in the leaf apoplast at moderate stages of Mn toxicity to better understand the sequence of events leading to leaf injury by Mn excess and specific mechanisms involved in Mn tolerance.

Visible Mn toxicity symptoms (brown spots) were detectable only after 3 days of elevated Mn supply of 10 µM Mn. Among the most sensitive responses of the leaf tissue was a steep increase in the Mn concentration of the apoplastic washing fluid (AWF), an enhanced callose formation, and a higher NADH-*per*oxidase activity in the AWF. This increase in NADH-*per*oxidase activity could be attributed to both a slightly enhanced release of proteins into the AWF and a slightly higher specific activity. An enhanced guaiacol-POD activity could be observed only after an increase in the density of brown spots.

On the basis of the presented results the following sequence of events in the expression of Mn toxicity is proposed: (i) increase in apoplastic free Mn, (ii) callose formation and stimulation of apoplastic H_2O_2-producing peroxidase, (iii) activation of cell wall-bound and soluble H_2O_2-consuming PODs, (iv) oxidation of Mn(II) and phenolics leading to the formation of brown depositions.

INTRODUCTION

During the last 20 years, research on the physiology of Mn toxicity in the leaf tissue of cowpea (*Vigna unguiculata*) was mainly conducted with plants, which showed clear expression of visible Mn toxicity symptoms. The application of high Mn concentration during cultivation of cowpea plants in hydroponics induces a rapid formation of brown spots, followed by chlorosis and necrosis. The occurrence of visible symptoms is preceded and accompanied by an early formation of callose formation (Wissemeier and Horst, 1987; Fecht-Christoffers et al., 2003b), the oxidation of ascorbic acid in the apoplast and symplast (Fecht-Christoffers et al., 2003a, d), and the activation and release of H_2O_2-producing and consuming *per*oxidases into the leaf apoplast (Fecht-Christoffers et al.,

2003b, c). Furthermore, pathogenesis related-like (PR-like) proteins (Fecht-Christoffers et al., 2003b), organic acids and phenolic compounds are excreted into the leaf apoplast (Horst et al., 1999; Fecht-Christoffers et al., 2003c). The release of the proteins and low molecular weight compounds by excess Mn reflects Mn sensitivity rather than Mn tissue tolerance. With prolonged Mn treatment, the responses of the leaf tissue to Mn excess bare similarities to "programmed cell death " (PCD). PCD is generally controlled by internal (genetically programmed) and external factors (induction of "hypersensitive response" by pathogens), and characterized by a broad range of physiological processes (Heath, 2000; Rao et al., 2000; Mittler, 2002; Thomas et al., 2003; Yoshida, 2003). Therefore, investigations on development and avoidance of Mn toxicity at a late stage of the toxicity cycle will display a mixture of Mn-induced primary and secondary physiological effects in the leaf tissue.

To expand our knowledge about physiological processes in cowpea specifically induced by Mn excess a moderate and slow development of Mn toxicity was induced in the Mn-sensitive cultivar TVu 91. A range of physiological and biochemical parameters as affected by Mn excess were monitored over a period of 6 days.

MATERIAL AND METHODS

Plant material and cultivation

Plants of cowpea (*Vigna unguiculata* (L.) Walp.), cultivar TVu 91, were grown hydroponically in a growth chamber under controlled environmental conditions (Fecht-Christoffers et al., 2003c). After preculture for at least 14 days, the Mn concentration in the nutrient solution was increased to 10 μM Mn ($MnSO_4$) for 1, 2, 3, 4, 5 or 6 days. Control plants received 0.2 μM Mn continuously. All plants were harvested at the same day. The nutrient solution was changed two to three times a week to avoid nutrient deficiencies.

Quantification of Mn toxicity symptoms

Leaf discs (1.54 cm^2) were cut out at the base, middle and tip of the trifoliate leaf and incubated in EtOH for at least 3 days. Numbers of brown spots on decolourised leaf discs were counted. The density of brown spots per square cm leaf area was calculated.

Extraction of Apoplastic Washing Fluid (AWF)

Apoplastic washing fluid (AWF) was extracted by a vacuum infiltration/centrifugation technique. Leaves were cut from plants, weighted and infiltrated with demineralised water (dH_2O). For this, leaves were transferred into vacuum resistance filtering flasks (DURAN®) filled with dH_2O. The pressure was reduced to 35 hPa (1 min) by using a water jet pump followed by slow relaxation for 2 min. Leaves were removed, dry blotted and were weighted again. AWF was recovered by centrifugation at 1324 x g for 5 min at room temperature.

Determination of the protein concentration in the AWF

Protein was quantified by Coomassie blue staining according to Bradford (1976).

Electrophoresis: 1D BN-PAGE

For purification of AWF proteins, centrifugal concentrators with a molecular weight cut-off (MWCO) at 5 kDa (Vivaspin 6, Vivasience, Hannover, Germany) were used. Samples volumes of 4 ml were reduced to 100 µl by centrifugation at 3000 g, 4°C for 1-2 h. AWF filtrates were removed and protein concentrates were washed with double demineralised water (ddH_2O) at 4°C.

Gel electrophoresis was carried out in Protean II Xi cells (gel dimension 20 x 18 cm) from Bio-Rad (Munich, Germany). Protein samples were combined with a Coomassie-blue solution (5% Serva Blue G, 750 mM aminocaproic acid). Gel pockets were pretreated for at least 30 min with buffer solution (50 mM Tricine, 15 mM Bis-Tris, 0.02% Coomassie-250G, pH 7, 4°C). Samples (100 µl) were loaded onto a native acrylamid gel with a 4% acrylamide stacking gel and a 12% to 20% gradient separation gel. Running conditions were selected as follows: (A) 45 min, 100V, max 15 mA, 4°C; (B) 9-10 h, 8 mA, max 500 V, 4°C. After electrophoresis the gel was soaked in 20 mM guaiacol, 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6) and 0.03% H_2O_2 to detect peroxidases in the gel.

*Determination of NADH- and guaiacol-*per*oxidase activity*

For measurement of NADH-*per*oxidase activity, samples were combined with 0.3 mM NADH, 1.6 mM p-coumaric acid and 16 mM $MnCl_2$ in 100 mM sodiumacetate buffer, pH 5. The activity was measured at λ=340 nm using a microplate reader (µQuant, BioTek Instruments GmbH, Neufahrn, Germany). For calculation of enzyme activities, the molar

extinction coefficient ε= 4.2378 mM^{-1} was used. Guaiacol-POD activities in the AWF were determined spectrophotometrically at λ=470 nm (UVIKON 943, BioTek Instruments GmbH, Neufahrn, Germany) by following the H_2O_2-depending oxidation of guaiacol. Samples were mixed with guaiacol solution (20 mM guaiacol in 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6) and 0.03% (w/w) H_2O_2). Enzyme activities were calculated using the molar extinction coefficient ε= 26.6 $mM^{-1}cm^{-1}$.

Callose extraction and detection

For the measurement of callose formation four leaf discs (150 mg fresh weight) were cut out of the leaf and fixed in ethanol. After 3 days ethanol was replaced by demineralised water (dH_2O) and incubated over night. Leaf discs were homogenized in 1 ml 1M NaOH and homogenates were incubated for 15 min at 80°C in a water bath. After centrifugation at 13000 x g for 5 min, 200 µl of the supernatant was mixed with 600 µl of the anilin mix [0.59 M glycine buffer (pH 9.5), 0.21 M HCl and 0.04% (w/v) anilin blue] and incubated in a 50°C water bath for 20 min. After cooling down the samples to room temperature, the callose concentration was measured by detecting the fluorescence at excitation wavelengt/bandpath 400nm/30nm, emission wavelength/bandpass 485nm/40nm with a microplate reader (FL_x800, BioTek-Instruments, Neufahrn, Germany). Control measurements were done with glycine-HCl-buffer solution without anilin blue. For the calculation the molar extinction coefficient ε=9.59 ppm^{-1} was used

Measurement of phenols in the AWF

AWF (50 µl) was combined with 350 µl ddH_2O and 50 µl Folin-Denis solution (Merck, Darmstadt, Germany). After 3 min incubation, 100 µl saturated Na_2CO_3 solution was added. After 1 h incubation and centrifugation (1 min, 5000 x g), absorption was measured at λ=725 nm in a microplate reader (µQuant, BioTek Instruments GmbH, Neufahrn, Germany). Concentrations were calculated in relation to p-coumaric acid standard solutions.

Mineral analysis

For detection of Mn in the bulk-leaf tissue centre ribs were cut out of the middle leaflets of trifoliate leaves and 0.5 – 1 g leaf tissue were dried at 65°C till constant weight. Drying was followed by dry ashing (480°C, 8h) and dissolving the ash in 6 M HCl with 1.5%

(w/v) hydroxylammonium chloride and dilution (1:10) with ddH_2O. AWF was diluted 1:10, whereas HCl and hydroxylammonium chloride was added to give final concentration of 0.6 M HCl and 0.15% (w/v) hydroxylammoniumchloride. Measurements were carried out by optical emission spectrometry, inductively coupled plasma (Spektro Flame, Spectro, Kleve, Germany).

Statistical analysis

Experiments were carried out with 16 individual samples per treatment. Each sample was collected from leaves (leaf insertion II) of two cowpea plants. Regression analysis, analysis of variance and multiple comparisons of means (Tukey test) were carried out by SAS 8e (SAS Institute Inc., Cary, North Carolina, USA). Different letters indicate significant differences between mean values at $p<0.05$ (Tukey).

RESULTS

Increasing Mn supply during cultivation of cowpea plants caused a strong increase of the concentration of Mn in the apoplastic washing fluid (AWF) after the first day of treatment (Fig. 1). With increasing duration of the Mn treatment (Fig. 1A) the concentration continued to increase, whereas after 2 days of treatment, the ratio of Mn in AWF to total Mn tissue content decreased (Fig. 1B). Total Mn tissue contents reached a level of approximately 1.7 µmol Mn per g fresh weight after a period of 6 days of elevated Mn supply. Visible Mn toxicity symptoms (brown spots) were detectable after the 3rd day (Fig. 2A). Toxicity symptoms were only slightly expressed as shown by an average of only 1 spot per cm^2 leaf area. After 5 to 6 days, approximately 4 to 6 spots were detected per cm^2 leaf area.

As an additional parameter of Mn toxicity, the Mn-induced formation of callose was quantified (Fig. 2B). The callose formation was induced even more sensitively than the brown spots: it increased slightly as early as 1 day and significantly after 2 days of Mn treatment.

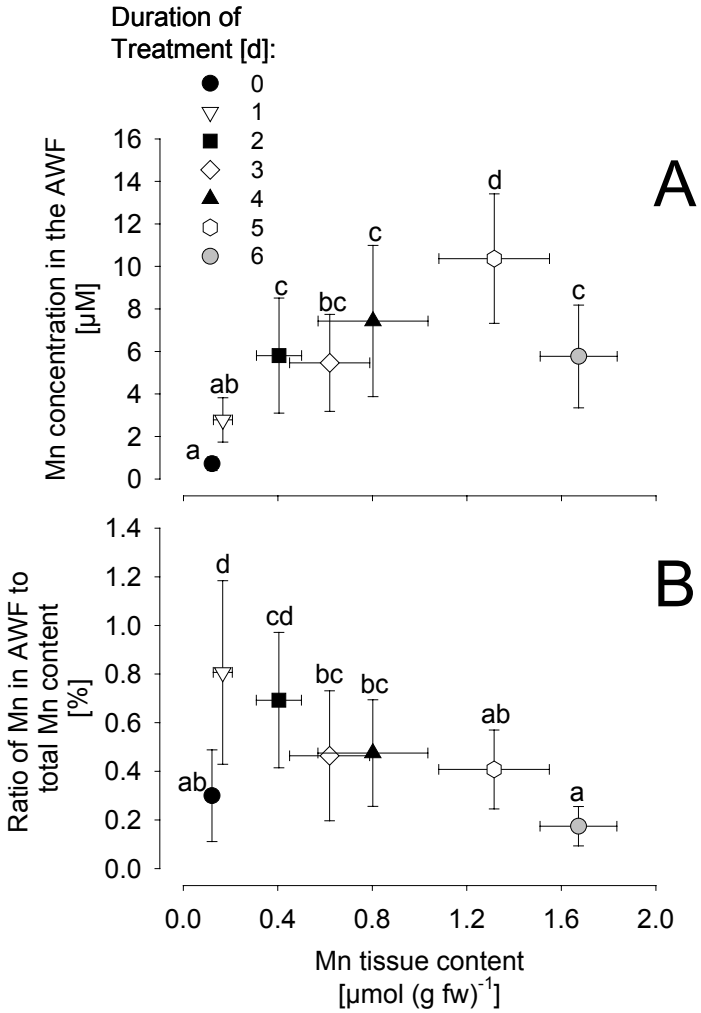

Figure 1 Relationships between Mn tissue contents and (A) the Mn concentration in the AWF and (B) the ratio of Mn in the AWF to total Mn tissue contents. Plants of cowpea cultivar TVu 91 (Mn sensitive) were treated with 10 µM Mn for 1, 2, 3, 4, 5 or 6 days. Control plants received 0.2 µM Mn continuously. n=16. Significant differences between mean values are indicated by different letters at $p<0.05$ (Tukey).

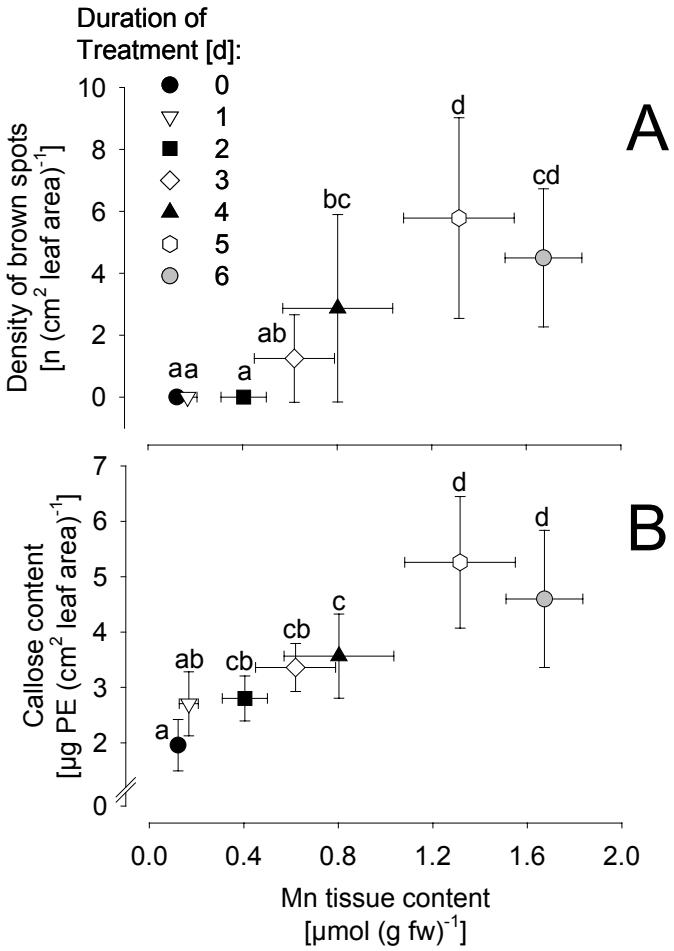

Figure 2 Relationships between Mn tissue contents (A) the density of brown spots of leaves and (B) the callose formation. Plants of cowpea cultivar TVu 91 (Mn sensitive) were treated with 10 µM Mn for 1, 2, 3, 4, 5 or 6 days. Control plants received 0.2 µM Mn continuously. n=16. Significant differences between mean values are indicated by different letters at $p<0.05$ (Tukey).

The activity of guaiacol-POD tended to increase after 4 days of Mn treatment, whereas a significant difference was measured not earlier than after 5 days (Fig. 3A). The activity of NADH-*per*oxidase was slightly enhanced on the 1st day and significantly increased on the 3rd day of Mn treatment (Fig. 3B).

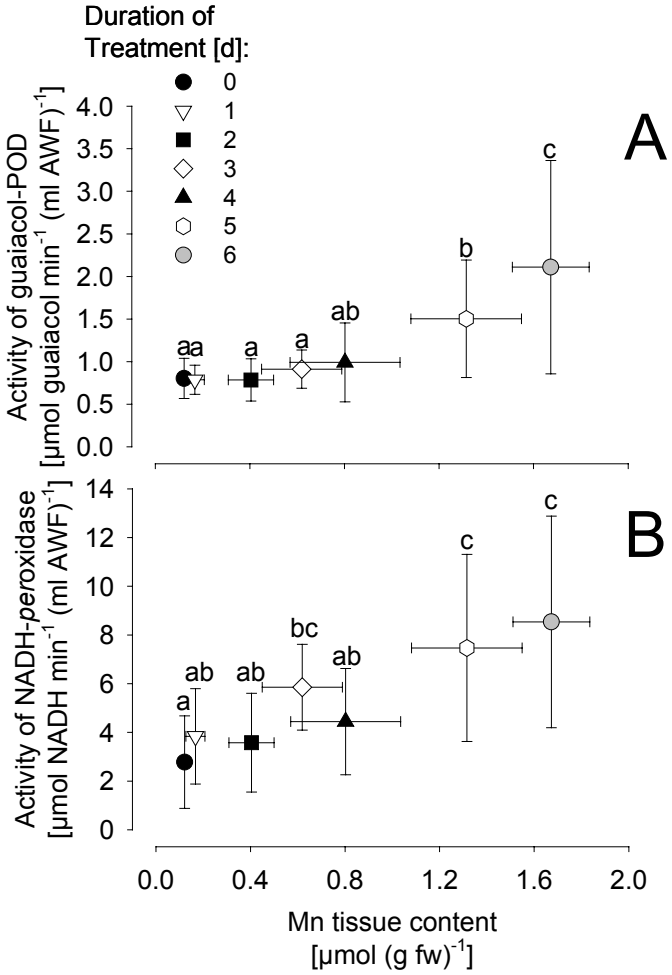

Figure 3 Relationships between Mn tissue contents and (A) the guaiacol-POD activity and (B) the activity of NADH-*per*oxidase. Plants of cowpea cultivar TVu 91 (Mn sensitive) were treated with 10 µM Mn for 1, 2, 3, 4, 5 or 6 days. Control plants received 0.2 µM Mn continuously. n=16. Significant differences between mean values are indicated by different letters at $p<0.05$ (Tukey).

A continuous increase in specific activities of both guaiacol-POD and NADH-*per*oxidase activities was observed with prolonged duration of increased Mn supply (Fig. 4). During the first 4 days of Mn treatment, no changes in total protein concentration in the leaf AWF were detectable. Only after the 5th and 6th day protein concentrations were slightly increased (Fig. 5).

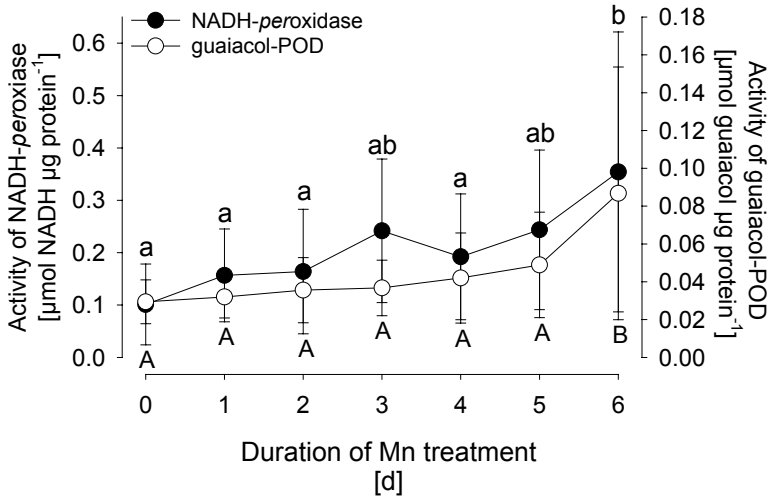

Figure 4 Effect of 6 days of elevated Mn supply on the specific activities of NADH-*per*oxidase and guaiacol-POD in the leaf AWF. Plants of cowpea cultivar TVu 91 (Mn sensitive) were treated with 10 µM Mn for 1, 2, 3, 4, 5 or 6 days. Control plants received 0.2 µM Mn continuously. n=16. Significant differences between mean values are indicated by different letters at p<0.05 (Tukey).

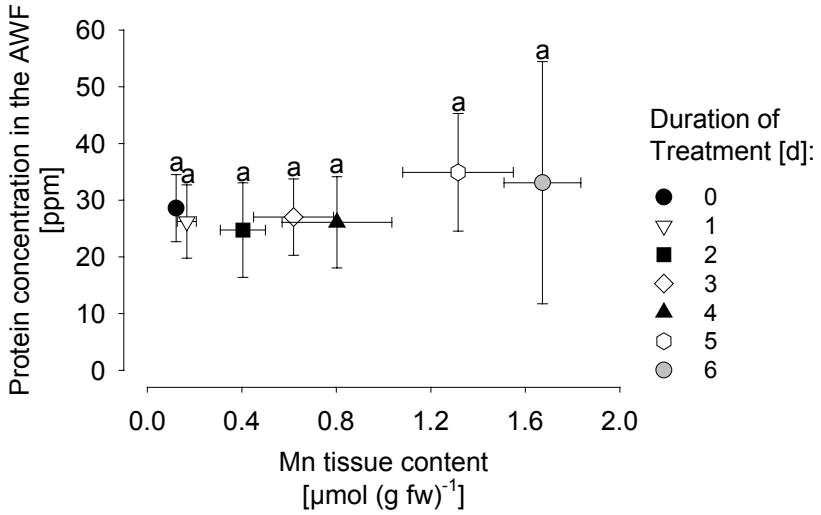

Figure 5 Relationship between Mn tissue contents and the protein concentration (BSA equivalents) in the AWF. n=16. Plants of cowpea cultivar TVu 91 (Mn sensitive) were treated with 10 µM Mn for 1, 2, 3, 4, 5 or 6 days. Control plants received 0.2 µM Mn continuously. n=16. Significant differences between mean values are indicated by different letters at p<0.05 (Tukey).

The significant increase of POD activities and the slight increase of protein concentration in the AWF on the 5th day of treatment is also displayed by a stronger expression of POD-isoenzymes and the appearance of further proteins in the leaf AWF (Fig. 6). The significant increase if POD activities after 5 days of treatment are therefore mainly caused by a release of PODs into the leaf apoplast.

A significant increase of phenol concentration in the leaf AWF was observed only on the 5th of treatment (Fig. 7).

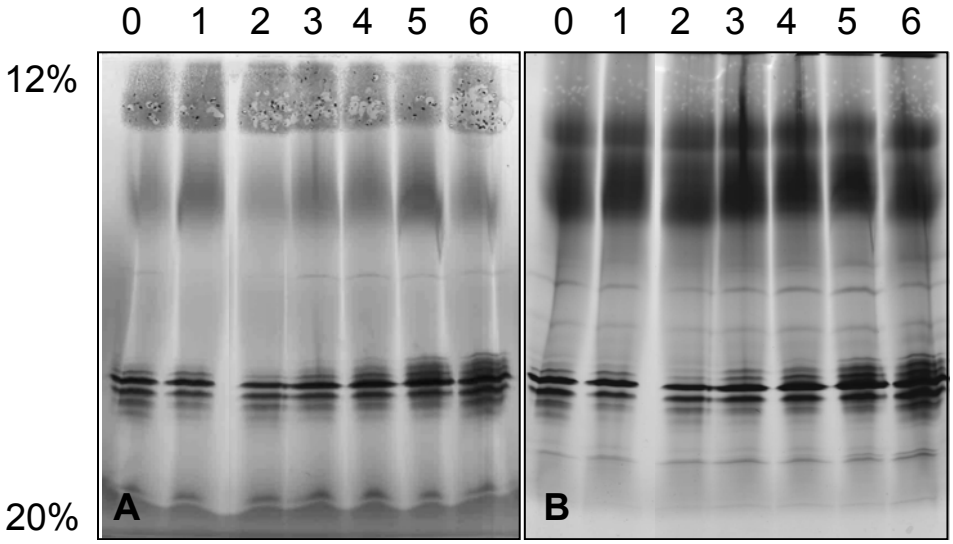

Figure 6 1D-electrophoresis resultion from the leaf AWF of Mn-sensitive cowpea cultivar TVu 91. AWF proteins were concentrated and 80 to 120 µg protein per lane were loaded onto the gel, approximately. Separation was carried out by Blue Native (BN)-PAGE using a 12% to 20% gradient gel. Peroxidases were detected by staining with 20 mM guaiacol + 0.03% H_2O_2 (A) and total protein with colloidal Coomassie Blue (B). Plants were treated with 10 µM Mn for 1, 2, 3, 4, 5 or 6 days (numbers on the top). Control plants received 0.2 µM Mn continuously.

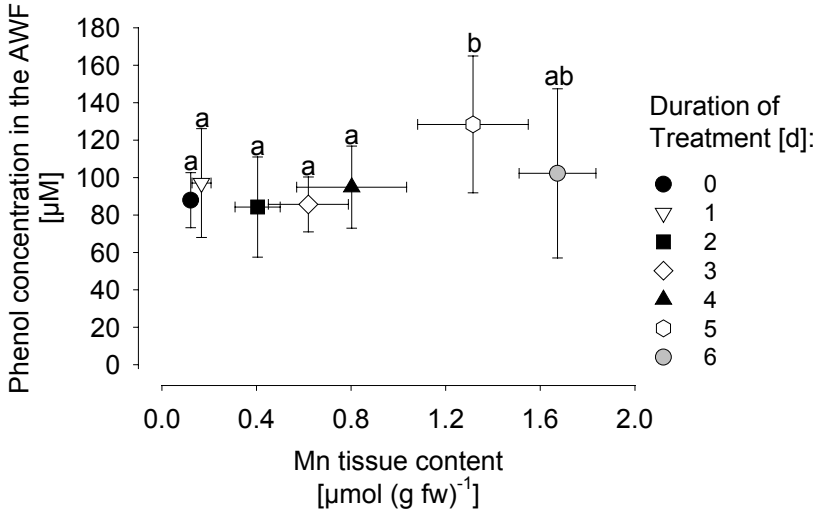

Figure 7 Relationships between Mn tissue contents and the phenol concentration (p-coumaric acid equivalents) in the AWF. Plants of cowpea cultivar TVu 91 (Mn sensitive) were treated with 10 µM Mn for 1, 2, 3, 4, 5 or 6 days. Control plants received 0.2 µM Mn continuously. n=16. Significant differences between mean values are indicated by different letters at $p<0.05$ (Tukey).

DISCUSSION

Enzymes (EC 1.11.1.7) of the peroxidase class III family are involved in a number of physiological reactions, e.g. lignification, suberization, auxin catabolism, wound healing caused by biotic and abiotic stresses, and defence against pathogen infection (Campa, 1991; Hiraga et al., 2001). A multitude of stresses affects the level of peroxidase activity in the plant tissue and therefore, plant peroxidases are frequently used as a biochemical marker for stress (Castillo, 1986). Particular apoplastic peroxidases respond sensitive to stress induced by ozone (Castillo et al., 1987; Peters et al., 1988; Ranieri et al., 2003), pathogen attack (Bestwick et al., 1998), excess Zn supply (Brune et al., 1994) and NaCl (Lin and Kao, 2001). A close relationship between the Mn-induced formation of brown spots, oxidation of Mn and phenolic compounds and the action of peroxidases was postulated (Wissemeier and Horst, 1992) and confirmed (Fig. 2 and 3; Fecht-Christoffers et al., 2003 a,c).

With the aim to study early physiological changes in the leaf apoplast by excess Mn, plants of the cowpea cultivar TVu 91 were treated with 10 µM Mn which was 5 times lower than in previous studies (Fecht-Christoffers et al., 2003b). This explains the moderate expression of Mn toxicity symptoms after the 3^{rt} day of treatment (1 brown spots per cm^2, Fig. 2) due to a lower Mn concentration in the AWF and a lower Mn content in the leaf tissue (Fig. 1) than after the application of 50 µM Mn (Fecht-Christoffers et al., 2003b) which lead after 3 days to 30 brown spots per cm^2 at Mn concentration of 10 µM in the AWF and Mn contents of 1.3 µmol Mn per g fresh leaf tissue. Comparable Mn levels were observed only at the 5th day of treatment with 10 µM Mn (Fig. 1).

At a moderate stage of Mn toxicity, no significant differences in the overall toxicity parameters could be observed between the individual samples. This is probably due to the fact, that the amount of affected tissue regions is very small compared with the healthy rest of the leaf tissue at this time.

However, increasing the Mn supply lead to an instantaneous increase in the free Mn concentration in the leaf apoplast, whereas the total Mn concentration increased more steadily over the whole Mn treatment period. From the ratio of AWF Mn to the total leaf Mn content (Fig. 1B) it appears that freshly fed Mn is preferentially translocated to the leaf apoplast. After this initial phase first the ratio and finally the absolute Mn concentration in AWF decreases indicating redistribution of Mn between the leaf compartments. This may be due to enhanced transport of Mn from the apoplast into the vacuoles (Maier, 1997).

Bound Mn also accumulates in cell walls (Memon et al., 1980; Iwasaki et al., 2002a), but approximately 80% of Mn is located in the symplast (Iwasaki et al., 2002a). Therefore, the cell wall is not a major site for Mn accumulation.

Peroxidases (POD) are proposed to catalyse the Mn-induced formation of brown depositions in the cell wall of the leaf tissue. However, there was only a slight increase of H_2O_2-consuming guaiacol-POD activity in the AWF simultaneously with the occurrence of first brown spots (Fig. 3A). This does not suggest a strong role of free apoplastic guaiacol-POD in the browning of the cell walls. Since cell wall-bound guaiacol-PODs are also activated by Mn treatment (Fecht-Christoffers et al., 2003a), they might contribute more directly to the formation of brown spots. In comparison to the guaiacol-POD the activity of H_2O_2-producing NADH-*per*oxidase in the AWF appeared to react more sensitively to Mn excess (Fig. 3B). There was a consistant increase in activity from the 1st day of Mn treatment on. Also, the consistently (although not significantly) higher specific activities of NADH-*per*oxidase compared to guaiacol-POD (Fig. 4) suggest an early Mn-induced stimulation of NADH-*per*oxidase. These results indicate differential sensitivities of apoplastic H_2O_2-producing and H_2O_2-consuming PODs in the apoplast to excess Mn (Fig. 4). A stronger stimulation of NADH-*per*oxidase than of guaiacol-POD was also observed in the AWF of plants, treated with 50 µM for a period of 4 days (Fecht-Christoffers et al., 2003c). Since exclusively NADH-*per*oxidase is directly influenced by Mn *in vitro* (Fecht-Christoffers et al., 2003c), increased Mn concentration in the AWF might stimulate the H_2O_2-producing POD directly *in vivo*.

It was shown that apoplastic H_2O_2-producing PODs respond earlier to stimuli than H_2O_2-consuming PODs. For instance, an increase in Mn^{2+}-2,4-dichlorophenol-stimulated NAD(P)H-*per*oxidases was observed 1h after inoculation of lettuce with bacterial strains, while a slight increase of guaiacol-POD was observed only after 2h (Bestwick et al., 1998). In the leaf apoplast of sunflower, particular ionically cell wall-bound NADH-*per*oxidases were significantly activated after 30 min of ozone exposure, whereas cell wall-bound and free movable syringaldazine-PODs were activated only after 2 h (Ranieri et al., 2003). Gaspar et al. (1985) and Castillo (1986) postulated a generalized two-step control of peroxidases in plant responses to different physical and chemical stimuli. The early and rapid stimulation of constitutive (basic) peroxidases was attributed to demasking effects by the involvement of phenolic compounds and Ca^{2+}. Phenolic compounds have an ambivalent effect on *per*oxidases. In cowpea, the activity of NADH-*per*oxidase is significantly stimulated by p-coumaric acid (Fecht-Christoffers et al., 2003c), but phenolic

compounds are also inhibiting NADH-*per*oxidases (Pedreño et al., 1987). The behaviour of phenolic compounds in the peroxidase cycle (Yokota and Yamazaki, 1977) is probably dependent on the reduction potential for phenoxy radical formation (Hauser and Olsen, 1998). However, the potential inhibiting effect of phenolic compounds on NADH-*per*oxidases was proposed to avoid Mn toxicity in cowpea (Fecht-Christoffers et al., 2003c). Since no statistically significant changes in total phenol concentration were detected at early stages of Mn toxicity (Fig. 5), only minor changes in phenol compositions in the apoplast might influence the functionality and activity of NADH-*per*oxidase.

The release of peroxidases (basic and acidic) was considered as a second step in response to stress stimuli (Gaspar et al., 1985). Ca^{2+} controls peroxidase activities and the secretion of peroxidases (Sticher et al., 1981; Castillo et al., 1984; Gaspar et al., 1985; Hu et al., 1987). While a direct effect of Ca^{2+} on peroxidase activities was demonstrated by Castillo et al. (1984) and Sato et al. (1995), Pedreño et al. (1989) postulated additionally a more indirect influence of Ca^{2+} on apoplastic peroxidases. Significant changes in the Ca^{2+}/H^{+} ratio were considered to induce modifications of apoplastic peroxidase activities due to significant changes in the local pH of cell walls. Indeed, small variations in pH values are shown to influence the activity of POD isoenzymes markedly (Sato et al., 1995; Otter and Polle, 1997; de Marco et al., 1999). A shift of the apoplastic pH from the acid to neutral or basic range was considered to be responsible for increasing POD-catalysed H_2O_2-formation (Bolwell et al, 1995). The alkalinization of the extracellular space is a characteristic event of the "oxidative burst" (Bolwell et al., 1995; Bolwell et al., 2002). In this work, changes in apoplastic pH were not investigated. Therefore, one can only speculate about an early Mn-induced change in the apoplastic pH causing an activation of NADH-*per*oxidase and enhanced H_2O_2-formation.

Callose [(1→3)-β-1,3-glucan] is a an extracellular structural polymer in higher plants (Delmer, 1987). Since a number of biotic and abiotic factors induce formation of callose, its detection is a reliable indicator for stress (Kauss, 1987). Particularly for the estimation of metal-induced injury of plant tissues, callose formation serves as a sensitive parameter (Horst et al., 1997; Kartusch, 2003). The formation of callose is also a sensitive and reliable parameter for Mn toxicity. Microscopic investigations showed callose depositions, which were localized isolated or close to brown depositions. The 20 times higher density of callose deposition than of brown spots (Wissemeier and Horst, 1987) and the early increase in total callose content (Fig. 2B) indicate an early influence of Mn on the plasma membrane localized β-1,3-glucan synthase. Divalent cations e.g. Mg^{2+}, Sr^{2+}, Ca^{2+} and

Mn^{2+} were shown to stimulate the enzyme directly (Morrow and Lucas, 1986), whereas Kartusch (2003) postulated a metal-mediated "Ca^{2+} signature" initiating a signal transduction chain reaction. Indeed, various elicitors induce callose formation accompanied by a rapid K^+ efflux and a temporally enhanced net Ca^{2+} influx. Since β-1,3-glucanase has a requirement for Ca^{2+}, the increase of cytoplasmic Ca^{2+} was proposed to trigger callose synthesis (Kauss et al., 1990).

CONCLUSIONS

On the basis of the presented results the following sequence of events in the expression of Mn toxicity is proposed:

(i) increase of Mn concentrations in the AWF

(ii) callose formation and stimulation of apoplastic H_2O_2-producing peroxidase

(iii) activation of cell wall-bound and soluble H_2O_2-consuming PODs

(iv) oxidation of Mn(II) and phenolics leading to the formation of brown depositions

(v) release of stress-related proteins into the apoplast.

This sequence may be initialised by an increase in apoplastic free Mn which could switch on a Ca^{2+}-dependent signalling pathway, accompanied by callose formation and an alkalinization of the apoplast. The alkalinization may shift the normally acid apoplastic pH to a higher pH appropriate for the action NADH-*per*oxidase producing H_2O_2. H_2O_2 might serve as a third or fourth messenger. Alternatively, the increased apoplastic Mn concentration could stimulate NADH-*per*oxidase directly. The elevated H_2O_2 could serve as a second messenger, mediating protein phosphorylation and the activation of transcription factors (Mittler, 2002; Neill et al., 2002) finally leading to the release of pathogenesis-related proteins (including PODs).

GENERAL DISCUSSION

In cowpea, first visible symptoms of Mn toxicity occur in physiologically older leaves by the expression of small dark brown spots, followed by necrosis, chlorosis and leaf shedding. These brown spots consist of local accumulations of oxidized Mn and oxidized phenolic compounds presumably in cell walls of the epidermis (Horst, 1988; Wissemeier and Horst, 1992). The oxidation of phenolic compounds and Mn by H_2O_2-consuming peroxidases (PODs) was suggested as key reaction leading to brown depositions and finally Mn toxicity. During catalysis, reactive intermediates, e.g. phenoxyradicals and Mn^{III} are presumably formed which were considered as primary phytotoxic agents causing leaf injury and finally leaf death. Since brown depositions occur in the cell walls, the leaf apoplast was suggested to be the most important compartment for development of Mn toxicity. Cowpea cultivars show considerable differences in the expression of Mn toxicity symptoms at elevated Mn tissue contents. The mechanisms of Mn toxicity and Mn tissue tolerance are not yet fully understood. The presented work focussed on the physiology of the leaf apoplast and and its modifications by excess Mn. In the following section, the most important results and conclusions are summarized and discussed in an integrated way.

The role of H_2O_2-consuming PODs in Mn toxicity

The presented data confirm a strong stimulation of H_2O_2-consuming and phenol-oxidizing PODs in the leaf tissue by excess Mn. Activities of cytoplasmic, cell wall bound and especially water soluble PODs from the leaf apoplast were significantly increased by Mn treatment (chapter 1). A close relationship between Mn tissue contents and the activity of PODs in the AWF was demonstrated (chapter 1, 2, 3, 4). The resolutions of Blue-Native (BN)-polyacrylamidgelectrophoresis (PAGE) display a strong release of peroxidase isoenzymes into the leaf apoplast at advanced stages of Mn toxicity (chapter 2). Several studies were focussed on the sequence of events in the development of Mn toxicity in order to improve the understanding of the specific relationship between the expression of Mn toxicity and the function of PODs (chapter 1, 2, 5). In response to relatively high Mn supplies (50 µM $MnSO_4$), brown spots were rapidly formed and POD activities in the AWF increased simultaneously with the appearance of these first visible toxicity symptoms. These results suggested the H_2O_2-consuming and phenol-oxidizing POD as the key enzyme in the expression of Mn toxicity. But subsequent experiments resulted in a different picture (chapter 5). Concentration on early Mn-induced physiological changes in

the apoplast by moderately high Mn supply (10 µM) revealed a rather late response of PODs in the AWF. The application of ascorbic acid, which was proposed to enhance Mn tolerance, suppressed POD activities significantly but the density of brown spots was only slightly affected (chapter 4). Therefore, the role of AWF PODs in the initiation of the formation of brown depositions is questionable. Since PODs are also abundantly bound to the cell wall of the cowpea leaf tissue and stimulated by excess Mn (chapter 1) cell wall-bound rather than free PODs are primary candidate enzymes responsible for the Mn-induced brown depositions in the leaf apoplast.

*The role of H_2O_2-producing NADH-*per*oxidase in Mn toxicity*

Several enzymes are catalysing the formation of H_2O_2 e.g. NADPH-oxidase, oxalate-oxidase (Bolwell et al., 1995) and diamine/polyamine-oxidase (Slocum and Furey, 1991; Yoda et al., 2003). In the apoplast, peroxidases are also involved in the formation of H_2O_2 needed for lignification or contributing to the "oxidative burst". NADH is oxidized in a complex sequence of redox reactions, accompanied by the formation of $O_2^{\cdot-}$ and $^{\cdot}OH$ (Liszkay et al., 2003). Although NADH is only rarely detected in the apoplast (Shinkle et al., 1992), it is considered the most probable substrate for H_2O_2-producing PODs *in vivo*. Since NADH is membrane-impermeant (Lin, 1982) it is either provided in the apoplast by cell-wall associated malate dehydrogenase (Gross et al., 1977). The H_2O_2-producing aspect of PODs might be crucial for the development of Mn toxicity, because (i) Mn stimulates NADH oxidation *in vitro* (chapter 3), (ii) NADH-*per*oxidase activities and the potential H_2O_2 formation are strongly enhanced in the leaf AWF of Mn-treated plants (chapter 2, 3) and (iii) an early increase of the specific enzyme activity in the AWF was observed at a moderate expression of Mn toxicity (chapter 5).

Phenolic compounds are strongly influencing the functionality of NADH-*per*oxidase by stimulating and inhibiting NADH oxidation. Particular the oxidase-peroxidase cycle is delayed by the addition of several phenolic compounds (Pedreño et al., 1987) and in the presence of non-enzymatic compounds in the AWF from the Mn-tolerant cowpea cultivar TVu 1987 (chapter 3). First approaches to characterize the phenol composition in the leaf AWF indicate genotypic differences in phenol composition of the AWF (chapter 3). Since an early Mn-induced release of phenolic compounds into the leaf apoplast could not be found, a constitutive genotypic difference in phenol composition might determine Mn

excess-enhanced H_2O_2 formation, the development of Mn toxicity, and genotypic differences in Mn tolerance.

The role of ascorbic acid in Mn toxicity

The role of ascorbic acid (AA) in Mn toxicity is discussed in chapter 1 and 4. The concentration of AA and the ratio of AA/(AA+DHA) in the leaf apoplast were significantly decreased by Mn treatment. A decrease of AA content and ratio of AA/(AA+DHA) in the leaf tissue was only observed along with a strong expression of Mn toxicity symptoms. The presented data suggest the induction of oxidative stress by Mn excess in the leaf apoplast very similar to the reported responses to ozone exposure. AA might act in the first line of defence, but did not prevent Mn-induced leaf injury at high stress levels. Particular in older leaves, the concentration of AA, the regeneration and the transport AA into the apoplast through the plasma membrane are presumably limited and insufficient to fully prevent Mn-induced leaf injury. Experiments with four common bean cultivars, differing in ozone tolerance and AA concentrations in the leaf apoplast, did not reveal a clear relationship between the capability to maintain a high availability of AA in the apoplast and genotypic Mn tolerance. The results suggest that the capability to maintain high AA levels in the leaf might contribute to delay Mn-induced leaf injury. But this can only partially explain genotypic differences in Mn tolerance.

Effect of excess Mn on the apoplast proteome

A detailed study of the apoplast proteome by two-dimensional resolution of total soluble proteins of the apoplast revealed a substantial release of proteins into the leaf apoplast at excess Mn. Peroxidases and several pathogenesis related-like (PR-like) proteins e.g. chitinase, glucanase, thaumatin-like proteins, and PR-like proteins class I, were identified by nano LC-MS/MS (chapter 2). The deployment of PR and PR-like proteins is regulated transcriptionally and contributes to a number of physiological responses following pathogen infection (Fig. 1).

In general, the infection of plant tissues by pathogens may cause the rapid death of plant cells which is termed “hypersensitive response” (HR) leading to a restriction of the growth and distribution of the pathogen (Heath, 2000). The infection might also cause a “systemic acquired resistance” (SAR), an immunization-like reaction throughout the plant leading to

improved tolerance of further pathogen infections (Campbell et al., 2002). The HR is an example for "programmed cell death" (PCD) (Greenberg, 1997).

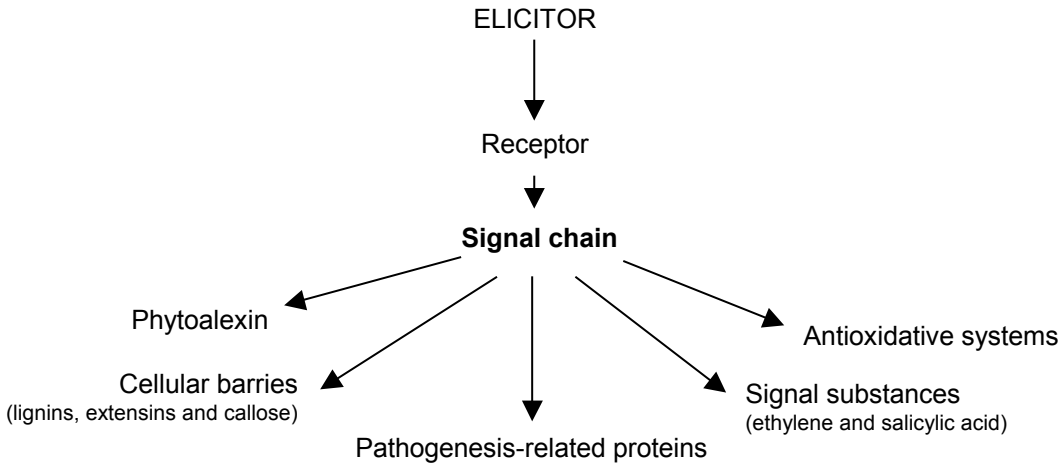

Figure 1 General scheme for the action of fungal elicitors (from Sandermann et al., 1998)

The term PCD describes cell death which is genetically programmed, e.g. during senescence (Thomas et al., 2003; Yoshida, 2003), but does not reveal the mechanism by which these cells die (Greenberg, 1997). Various receptors and signal chains are considered as being involved in both HR and SAR (Greenberg, 1997; Heath, 2000; Rao et al., 2000). Ozone exposure induces plant responses, which are comparable to those of pathogen infections (Rao et al., 2000). This is presumably based on the induction of similar or overlapping signalling pathways (Moeder et al., 1999). One of the earliest events in response to pathogen infection and ozone is the generation of reactive oxygen species (ROS or AOS, "oxydative burst"; Lamb and Dixon, 1997; Sandermann et al., 1998; Wohlgemuth et al., 2002). The formation of ROS, particular of H_2O_2 was discussed as signalling molecule triggering HR (Levine et al., 1994; Tenhaken et al., 1995) and SAR (Chen et al., 1993). Various abiotic and biotic stresses, e.g. high light intensities, UV irradiation, ozone, temperature extremes, dehydration, wounding, pathogen challenge and elicitors are reported to enhance H_2O_2 levels in plant tissues (Neill et al., 2002), and various abiotic factors, e.g. ozone, UV irradiation, salt, temperature, wounding and heavy metals cause the expression of PR-like proteins (see chapter 2 and citations within). Therefore, it is likely that a number of different stimuli induce comparable responses within the tissue, and especially H_2O_2 might mediate cross-talk between signalling pathways and might contribute to the phenomenon of `cross-tolerance´ (Bowler and Fluhr, 2000).

The ozone and pathogen-induced physiological changes in the plant tissue displayed in Fig. 1 are also partially induced by excess Mn. Elevated phenol contents in leaf tissues (Langheinrich et al., 1992) and a release of phenolic compounds in the leaf apoplast of cowpea were reported (chapter 3). In cowpea, Mn excess causes the formation of callose and brown depositions consisting of oxidized phenolic compounds in cell walls (chapter 2 and 5). The expression of PR-like proteins was already mentioned (chapter 2) and the Mn-induced formation of ethylene was reported by Horst, 1988. The antioxidative system in the apoplast and cytoplasm is also significantly affected by Mn (chapter 1 and 4), and elevated H_2O_2 concentrations in the surface solution of washed intact leaf segments of cowpea cv TVu 91 was reported by Horst et al. (1999). Therefore, Mn might induce processes in the leaf apoplast, which are similar to the cascades of the "oxidative burst", HR, or SAR.

The sequence of Mn toxicity development and potential signal chains

Plants responses to biotic or abiotic stresses are often detectable within minutes or hours (Lamb and Dixon, 1997; Ranieri et al., 2003). In this work, experiments were conducted over a period of several days with sampling on a daily basis. At a moderate stage of Mn toxicity, no significant differences in the overall toxicity parameters could be observed between the individual samples (chapter 5). This may be due to the fact that Mn toxicity is expressed initially only in very localised leaf-tissue areas around brown spots in the epidermis. A more uniform response could be expeced in cell suspension cultures. This is supported by studies with soybean cell suspension-cultures showing early responses to excess Mn already after 4 h (Fig. 2). Soybean belongs to the plant species expressing Mn toxicity in a similar way as cowpea (Carter et al., 1975; Heenan and Carter, 1976; Wu, 1994). A sequence of Mn-induced changes in the incubation medium which represents the apoplast of the suspension cells, similar to the recorded changes in the leaf AWF of cowpea (see above) was observed: Mn excess induced a rapid induction of callose formation followed by elevated POD activities in the incubation medium (Fig.2). The formation of callose is the earliest physiological response to excess Mn measured in leaf tissues of cowpea. Since the callose sythesizing enzyme β-1,3 glucanase requires for Ca^{2+}, an increase of cytoplasmic Ca^{2+} was proposed to trigger callose synthesis (Kauss et al., 1990). Mn^{2+} may stimulate β-1,3 glucanase directly (Morrow and Lucas, 1986) or indirectly through triggering the cytosolic Ca^{2+} signal.

For H_2O_2-mediated signal transduction, Ca^{2+}-dependent and independent signal transduction pathways are discussed (Neill et al., 2002; Mittler, 2002). Both, the Ca^{2+}-dependent protein kinase (CDPK) and the Ca^{2+}-independent mitogen-activated protein kinase (MAPK) respond to receptors or sensors and catalyse the addition of phosphate groups to proteins, which activate or deactivate enzymes and modulate steps in the signal transduction cascades (Neill et al., 2002). Investigations on bivalent cation requirements of CDPK and MAPK clearly showed that Mn^{2+} could replace Mg^{2+}. The activity of CDPK was actually more stimulated by Mn^{2+} than by Mg^{2+} (Duan et al., 2003).

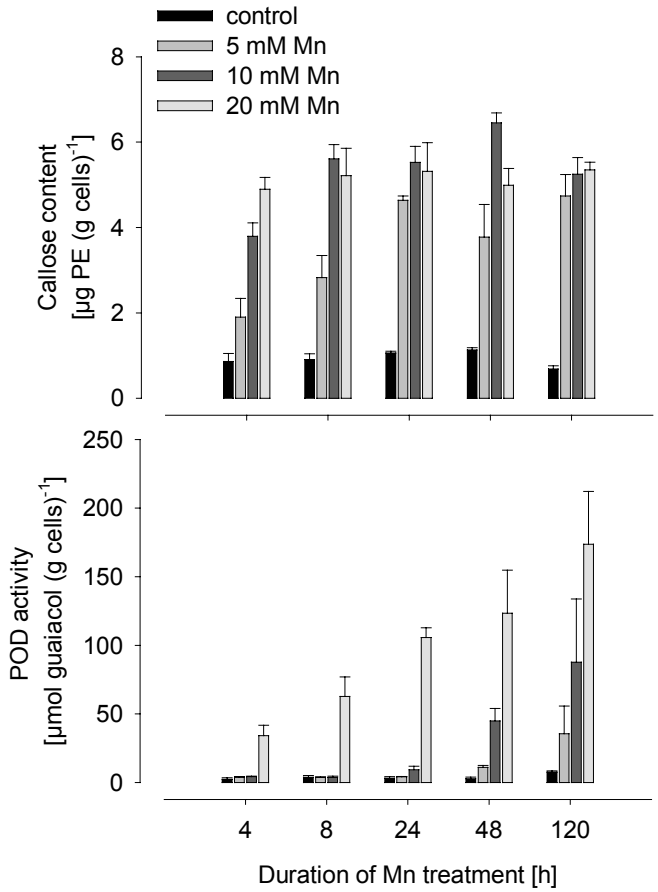

Figure 2 Effect of elevated Mn concentrations in the culture medium of soybean-cell suspension-culture on the callose formation and POD activity in the incubation medium. *Glycine max*. (L.) Merr. `Mandarin´ cell suspension-culture was cultivated in B5 medium at 27°C in the dark. For the study of Mn toxicity, cells were filtrated and washed three-fold with 2% (w/v) sucrose solution. Approximately 3 g cells were transferred in 100 ml 1:20 fold diluted B5 medium. Aliqots of 10 ml were incubated with 0, 5, 10 and 20 mM $MnSO_4$ for 4, 8, 24, 48 and 120 h at room temperature. Callose contents and POD activities of cells were detected as described by Fecht-Christoffers et al. (2003b).

Mn^{2+} also stimulated the phosphorylation of several membrane-associated phosphoproteins from barley roots (Reuveni and DuPont, 2001), and a class of membrane-associated

Arabidopsis protein kinases exhibit a preference for Mn^{2+} over Mg^{2+} (Schaller and Bleecker, 1993).

Due to the lack of detailed investigation on early physiological changes in the apoplast, a signalling pathway in response to high Mn supply is difficult to predict. The induction of callose might be a response to elevated H_2O_2 concentration in the leaf apoplast, caused by a stimulation of NADH-*per*oxidase. On the other hand, the steep increase in apoplastic free Mn^{2+} might induce a Ca^{2+} signature with following callose formation and alkalinization of the apoplast (Bolwell et al., 1995) thus shifting the apoplastic pH to the optimum for NADH-*per*oxidase catalysed formation of H_2O_2. In the latter case, H_2O_2 would act as a third messenger. Also, changes in the apoplast AA levels are involved in signal transduction from the apoplast to the symplast (discussed in chapter 4). Since no direct interaction of AA and Mn^{2+} was indicated, a decline of AA in the apoplast indicates the presence of other reactive species induced by excess Mn. Therefore, changes in the apoplastic redox stage reflected by changes in AA levels presumably serves only as third or fourth messenger. The release of PR-like proteins, phenolic compounds and organic acid into the apoplast is rather a late Mn-induced stress responses.

The proposed reactions in the leaf apoplast of cowpea by excess Mn are summarised and displayed in Fig. 3.

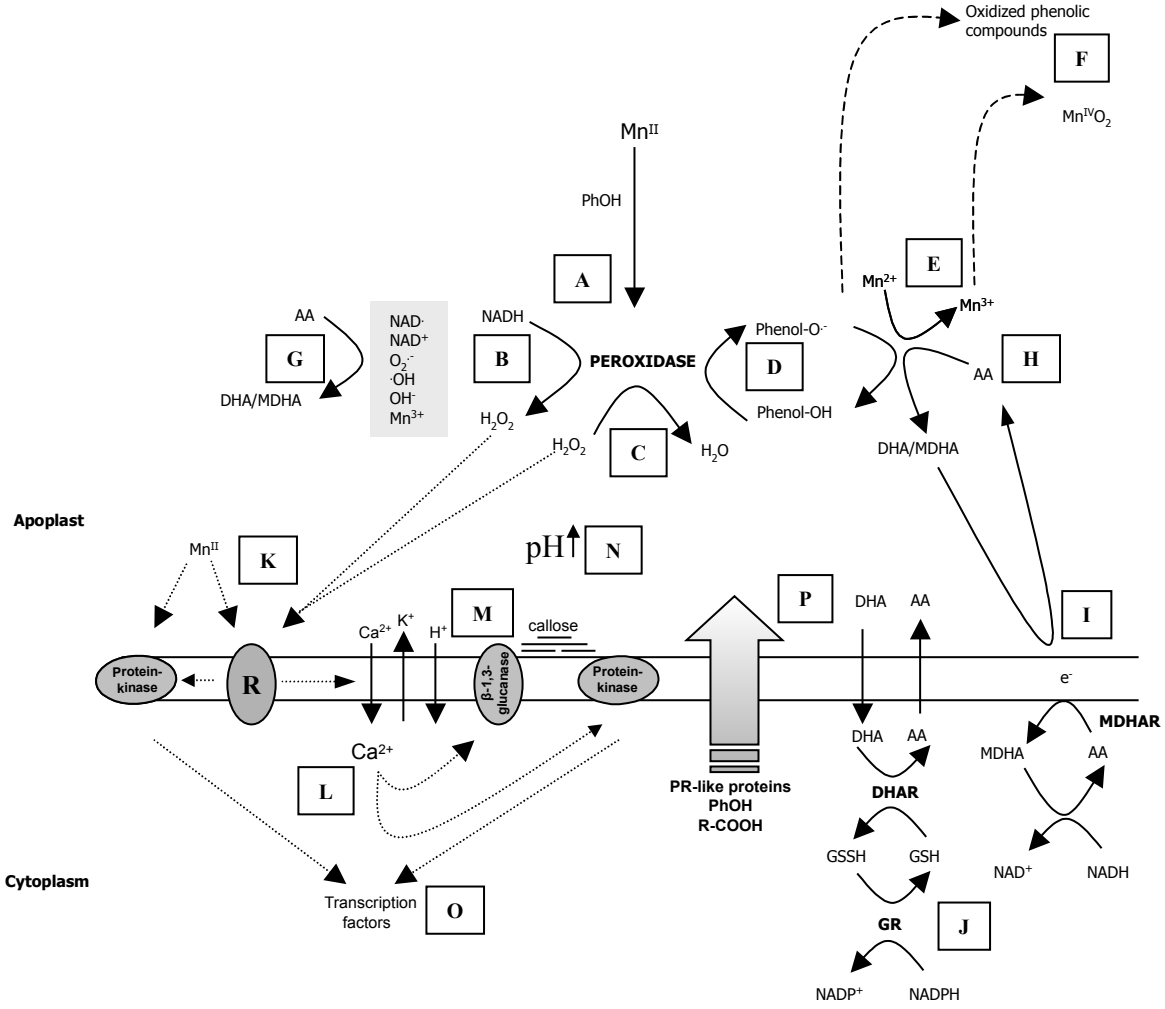

Figure 3 Proposed reactions in the leaf apoplast of Mn-sensitive tissues of cowpea (*Vigna unguiculata*). Peroxidases (PODs) are directly stimulated by elevated Mn concentrations in the leaf AWF (**A**). H_2O_2 is formed during the peroxidase-oxidase cycle (**B**) and consumed by the peroxidatic-peroxidase cycle (**C**). Intermediates of phenol oxidation (phenoxyradicals) (**D**) are oxidizing Mn^{II} causing the formation of Mn^{III} (**E**). Mn^{III} is oxidized to $Mn^{IV}O_2$, which accumulates together with oxidized phenolic compounds in the cell wall, causing the formation of brown depositions and spots (**F**). Ascorbic acid is oxidized in the peroxidase-oxidase (**G**) and in the peroxidatic cycle of POD (**H**). The primary oxidation product monodehydroascorbate (MDHA) is regenerated by monodehydroascorbate reductase (MDHAR) (**I**), or oxidized to dehydroascorbate (DHA), which is regenerated in the cytoplasm (**J**). Elevated concentrations of H_2O_2 in the AWF might act as a second messenger, stimulating a receptor and protein kinases (**K**), or Mn^{II} might stimulate the signalling pathway by stimulating receptors or protein-kinases (**K**). The hypothetic induction of a "Ca^{2+} signature" (**L**) might cause callose synthesis (**M**) and the alkalinization of the apoplast (**N**), which stimulates NADH-*per*oxidase. The induction of a signal cascade causes the activation of transcription factors (**O**), indicated by the expression of PR-like proteins (**P**).

OUTLOOK

The present work clearly points out that Mn induced processes at advanced stages of Mn toxicity are representing rather secondary physiological processes in the leaf tissue and particular in the leaf apoplast of cowpea. Therefore, further research should focus on the initial phase of Mn toxicity. The leaf apoplast is still an important compartment for development of Mn toxicity and tolerance. The sequence of Mn toxicity development is not yet clarified, but this work indicated two early responses to excess Mn: (i) the formation of callose, and (ii) the increase of NADH-*per*oxidase activity in the leaf apoplast. Both aspects are possible candidates for indicating or initiating first physiological responses to Mn excess. A more detailed kinetic study with emphasis on very early stages of Mn toxicity and a comparison of Mn-sensitive and –tolerant leaves (genotype and leaf age) are required.

The intensification of research on the role of NADH-*per*oxidase in Mn toxicity is recommended, because a strong and early stimulation by Mn was shown, presumable responsible for elevated levels of the signalling molecule H_2O_2 in the apoplast. For this, cell wall bound and membrane associated PODs have to be taken into consideration. The extraction, purification and the characterization by determination of molecular weight, pI values, pH optima, K_m and V_{max} values are fundamental experimental approaches. Since NADH-*per*oxidases are strongly affected by non-protein compounds in the AWF, the characterization and identification of these promoters and inhibitors is required. Phenolic compounds are possible candidates for the regulation of PODs. The cowpea cultivars TVu 91 and TVu 1987 showed different phenol compositions in the AWF, and this difference is proposed to be crucial for differences in Mn tolerance. The hypothesis could by verified by cross-wise combination of separated phenolic compounds from the AWF with purified peroxidases from the leaf apoplast, as described in chapter 3. If significant effects can be obtained, the identification of phenolic compounds should follow.

For the investigation on initial Mn induced steps in Mn toxicity, the influence of the whole plant system on the results of experiments has to be taken into consideration, as the amount of affected tissue is in general very small compared to the healthy rest of the plant. This is indicated by the results presented in chapter 5, where no significant differences could be observed during the first days of Mn treatment. In the leaves of cowpea, Mn toxicity occurred predominantly unevenly distributed, particular in the leaf

epidermis. But the AWF contains proteins, compounds and ions from the total apoplast, also from areas of the tissue which where not or less affected by excess Mn. Other experiments conducted with cell suspension cultures of soybean gave a more detailed picture of the sequence of Mn toxicity. A more homogeneous cell material will result in small variations between replicates (see general discussion) and this unhindered access to the "apoplast" will give new experimental opportunities. The application of enzyme inhibitors is a common approach to investigate signalling pathways and signal cascades, particular in relation to the "oxidative burst". Despite the missing specificity of some of these inhibitors, this application might give new insights in the initial cascade of Mn induced processes.

In the presented work, also a more general approach was selected by the investigation of the apoplast proteome. This proteomic approach has greatly improved our knowledge on Mn toxicity. Until now, only the total water-soluble proteins were investigated. To expand our understanding about Mn induced processes, the total apoplast proteome including cell wall bound proteins has to be characterized. For completion, early changes in the cytoplasmic proteome and changes of the transcriptome have also to be included into the experiments and will give new insights into Mn induced processes in the leaf tissue of Mn sensitive and Mn tolerant tissue. These approaches allow a more general view on Mn induced processes and systems in tissues and will give a chance to reveal physiological mechanisms involved in Mn toxicity and Mn tolerance.

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VERÖFFENTLICHUNGEN

INTERNATIONALE VERÖFFENTLICHUNGEN

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POSTERPRÄSENTATIONEN

Maier, P., Fecht., M., Horst, W.J. (1998) Bedeutung von Ascorbat für die Mangantoleranz bei Cowpea. Botaniker Tagung Bremen

Maier, P., Fecht., M., Horst, W.J. (1999) Peroxidase activity in the leaf apoplast is a sensitive marker for Mn toxicity and Mn tolerance in *Vigna unguiculata.* DFG-Tagung SPP 717, Bonn

Fecht, M., Maier, P., Horst, W.J. (2000) Peroxidase activity in the leaf apoplast is a sensitive marker for Mn toxicity and Mn tolerance in *Vigna unguiculata* (L.) Walp. Botaniker Tagung Jena.

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Fecht, M., Maier, P., Horst, W.J. (2001) Peroxidase activity in the leaf apoplast is a sensitive marker for Mn toxicity and Mn tolerance in *Vigna unguiculata* (L.) Walp. IPNC Hannover

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Fecht, M., Horst, W.J. (2001) Effect of Manganese treatment on the H_2O_2-concentration and generation by an NAD(P)H oxidizing system in the apoplast of Cowpea. DFG-Tagung SPP 717, Summer School, Berlin

Fecht-Christoffers M.M., Braun, H.P., Lemaitre-Guiller, C., Horst, W.J. (2003) Effect of Mn treatment on the protein composition in the leaf apoplast of cowpea (*Vigna unguiculata*). Festkolloquium 200 Jahre Justus Liebig, DGP-Tagung Giessen.

BUCHBEITRÄGE

Horst, J.W., Staß, A., Fecht-Christoffers, M.M. (2004) Mineral Element Toxicities - Aluminium and Manganese. In Hock, Elster (eds) Plant toxicology (eingereicht)

Fecht-Christoffers M.M., Maier P., Iwasaki K., Braun H.P., Horst W.J. (2004) The role of the leaf apoplast in manganese toxicity and tolerance in cowpea (*Vigna unguiculata* L. Walp.). Development in Plant and Soil Sciences, Kluwer Academic Publishers. (in Vorbereitung)

GASTVORTRÄGE

DFG-Tagung, 2001 Berlin „Einfluß von Mangan auf Redoxprozesse im Blattapoplasten und deren Bedeutung für die Mangan Gewebetoleranz bei cowpea (*Vigna unguiculata* L. Walp.)“

Workshop Pflanzenproteomics, 2003 Hannover „Physiologie von Mangan-Toxizität: Einfluß eines Mangan Überangebotes auf die Proteinzusammensetzung des Blattapoplasten von *Vigna unguiculata*“

Kolloqium Pflanzenernährung, Stuttgart-Hohenheim, 2003 „The physiology of Mn toxicity and Mn tolerance of *Vigna unguiculata*“

Workshop Pflanzenproteomics, 2004 Frankfurt „Effect of Manganese toxicity on the proteome of the leaf apoplast in cowpea (*Vigna unguiculata* L. Walp.)”

Auszeichnung für das Poster **„Effect of Mn treatment on the protein composition in the leaf apoplast of cowpea”** auf der Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung (DGP) 2003 in Giessen.

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