Ascorbic Acid, Thiamine or Salicylic Acid Induced Changes in Some Physiological Parameters in Wheat Grown under Copper Stress

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Abstract

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The fresh and dry weight of roots and shoots of wheat seedlings showed a marked decrease as the concentration of copper (Cu) was increased. The contents of pectin, cellulose, soluble carbohydrates, and phospholipids in the roots and shoots of test plants were significantly lowered with the rise of Cu concentration. However, hemicellulose, lignin, total lipids, glycolipids, sterols and proline of roots and shoots were raised under Cu stress. Cu treatment also induced the stimulation of soluble, total and cell wall associated proteins, other free amino acids and total carbohydrates in shoots and a slight reduction in roots. The adverse effects of Cu toxicity treatments on root and shoot growth were partially alleviated by the treatment of test plants with ascorbic acid, thiamine (vitamin B_1) and salicylic acid.

Keywords: ascorbic acid; vitamin B_1; carbohydrates; cell wall fractions; copper; lipid fractions; proline; proteins; salicylic acid; *Triticum aestivum* L.

Copper (Cu) is a heavy metal that is an essential microelement for plant metabolism. It participates in important biological reactions as an enzymatic cofactor (cytochrome c oxidase and amine oxidase) and as an electron carrier in oxidative phosphorylation and photosynthesis (Solomon & Lowery 1993). It can also be strongly phytotoxic at high concentrations (Rooney *et al.* 2006). In plants, the toxic effect of heavy metals includes interference with many metabolic reactions such as photosynthesis, respiration and protein synthesis. Cu exposures were also proved to cause more serious damage to the cell membrane and to reduce plant growth by producing reactive oxygen species in non-tolerant plants (Liu *et al.* 2004; Liu & Xiong 2005). Due to its widespread industrial and agricultural use, copper poisoning is a troublesome environmental problem. Despite decades of research (Dahmani-Muller *et al.* 2000; Brekken & Steinnes 2004), the data on remediation of heavy-metal toxicity and reduction of metal accumulation in plants are limited.

Ascorbate (vitamin C) is an essential antioxidant in the ascorbate-glutathione pathway, but it also protects enzymes that have prosthetic transition metal ions. Furthermore, it is a cofactor for many enzymes, including those involved in the cell wall synthesis, most notably in the hydroxylation of proline residues (Ishikawa *et al.* 2006). Moreover, alternative oxidase can be induced by H_2O_2 accumulation and, as ascorbate is involved in controlling the intracellular H_2O_2 level, this might provide the means for a concerted interaction to protect the cell against uncontrolled oxidation (Bartoli *et al.* 2006).

Thiamine is a B-complex vitamin that is produced in plants and microbes, including brewer's yeast (*Saccharomyces cerevisiae*; Burrows *et al.* 2000) and *Salmonella typhimurium* (Beck & Downs

1999). In addition to the important role of thiamine in plant biosynthetic pathways, limited information suggests that it can also function as an inducer of the accumulation of pathogenesis-related protein (PR) in a salicylic acid-dependent pathway and enhancement of disease resistance in tobacco, *Arabidopsis*, cucumber and rice (Malamy *et al*. 1996; Ahn *et al*. 2005). Moreover, the thiazole biosynthetic enzyme of yeast and *Arabidopsis* appear to be a dual functional protein with the role in thiamine biosynthesis and mitochondrial DNA damage tolerance (Machado *et al*. 1996, 1997). Although there is no direct evidence to support the hypothesis, the DNA damage tolerance associated with the thiazole biosynthetic enzymes of yeast and *Arabidopsis* may be due to the action of thiamine as observed in human lymphocytes in which the radiation-induced genetic changes can be protected by thiamine (Konopacka & Rogolinski 2004). Besides its physiological and genetic importance, priming by thiamine could be one of the most economical and effective resistances because the expression of defence-related mechanisms in the absence of pathogen requires the plant's metabolic investment necessary for growth or other fitness-related processes (Purrington 2000; Heil 2001; Van Hulten *et al*. 2006).

Salicylic acid (SA) has received much attention due to its association with economically important plant responses to diseases and other stresses. Detailed evidence implicates SA in PR gene expression, systemic acquired resistance, and hypersensitive response (Kunkel & Brooks 2002). SA also seems to be involved in responses to abiotic stresses, such as ozone (Sharma *et al*. 1996; Rao & Davis 1999; Koch *et al*. 2000), salt and osmotic stress (Borsani *et al*. 2001; Molina *et al*. 2002; Shim *et al*. 2003), UV-B (Surplus *et al*. 1998), drought (Senaratna *et al*. 2000; Nemeth *et al*. 2002), herbicides (Kim *et al*. 2003) and heat (Senaratna *et al*. 2000; Clarke *et al*. 2004). Stress-influenced developmental transitions, including flowering (Hatayama & Takeno 2003; Martinez *et al*. 2004), tuberization (Lopez-Delgado & Scott 1997), and senescence (Morris *et al*. 2000) may also involve SA.

Priming is one of the most efficient types of induced resistance because the metabolic investment of the plant for the constitutive activation of the defence system is reduced or prevented.

Additionally, the effects of exogenous vitamins (ascorbic acid and thiamine) on carbohydrates, proteins, proline, other free amino acids, glycolipids, phospholipids, sterols, total lipids and cell wall fractions (pectin, hemicellulose, cellulose, lignin and cell wall-associated protein) have not been examined in plants under heavy metal stress.

To address these issues, we chose wheat, the important and widely adapted food cereal in Egypt, as the experimental material to investigate whether two forms of water soluble vitamins (C and B_1) and salicylic acid confer protection against Cu toxicity in plant leaves.

MATERIAL AND METHODS

Five-day-old wheat (*Triticum aestivum* L.) seedlings were grown hydroponically in aerated Hoagland solution (Hoagland & Arnon 1950) in a greenhouse under natural light for 2 weeks. Each pot, which represents an experimental unit with four seedlings, was supplied with 400 ml distilled water. The design of the experiment was done with four replications. Copper treatment was performed by supplementing the nutrient solution with an increasing concentration of copper ions in the form of $CuSO_4$ (0, 5, 10, 20, and 40 mg/l Cu^{2+}) and the plants were left for 3 days. Some of the plants were pre-treated with 100 ppm ascorbic acid (AsA), thiamine hydrochloride (B_1) or salicylic acid as sodium salicylate added to the hydroponic solution for 1 day only. At the end of the experimental period fresh shoots and roots were then dried in an aerated oven at 70°C.

The cell wall fractionation was conducted according to Dever *et al*. (1968) and Selvendran and O'neill (2006). Contents of wall polysaccharides were determined by the anthrone sulphuric acid reagent using glucose as standard (Fales 1951). Prior to the fractionation of wall polysaccharides, proteins associated with the cell walls were extracted with 3M LiCl in Na-citrate or phosphate (10mM, pH 5.5) at 4°C for 48 h (Huber & Nevins 1979; Acebes & Zarra 1992). Extracted proteins were then quantified with the Folin's phenol reagent (Lowry *et al*. 1951). Lipids were extracted three times from dried plant organs (each extraction lasted for 24 h) with chloroform/methanol (2:1, v/v) at room temperature according to Navari-Izzo *et al*. (1989). Lipids (100 µl) were chromatographed on silica gel (silica gel G/60) plates using *n*-hexane-diethylether-glacial acetic acid (70:30:1) and the spots were made visible by I_2 vapour. According to Navari-Izzo *et al*. (1989) the total lipids (TPL)

were located at the origin of the chromatogram. They were scratched and redissolved in chloroform/methanol (2:1). Glycolipids and phospholipids were quantified via their sugar content that was estimated by the anthrone sulphuric acid method (described above) and regarded as an index of the glycolipid content. Phospholipids were determined in lipid extracts according to Johnson (1971). Lipid phosphorus was estimated by the molybdate blue colour (Woods & Mellon 1941). Phosphorus content was taken as an index of the phospholipid content. Total sterols were estimated according to the method described by Cook (1958). Proline was estimated by ninhydrin reagent (Bates *et al.* 1973). The absorbance of the fraction with toluene aspired from the liquid phase was read at 520 nm. Proline concentration was determined using a calibration curve.

Water extract was used for the determination of soluble carbohydrates, soluble protein and other free amino acids.

The anthrone sulphuric acid method (Fales 1951; Schlegel 1956) was used for the determination of carbohydrates. A calibration curve using pure glucose was constructed.

Other free amino acids were determined according to the method of Moore and Stein (1948). A calibration curve was constructed using glycine.

Proteins were estimated by Folin's reagent according to Lowry *et al.* (1951). A calibration curve was constructed using bovine serum albumin (BSA).

The data were subjected to one-way analysis of variance (ANOVA) using the SPSS statistical package. Duncan's multiple range test ($P < 0.05$) was used for a comparison of the means of four replications of three independent experiments.

RESULTS

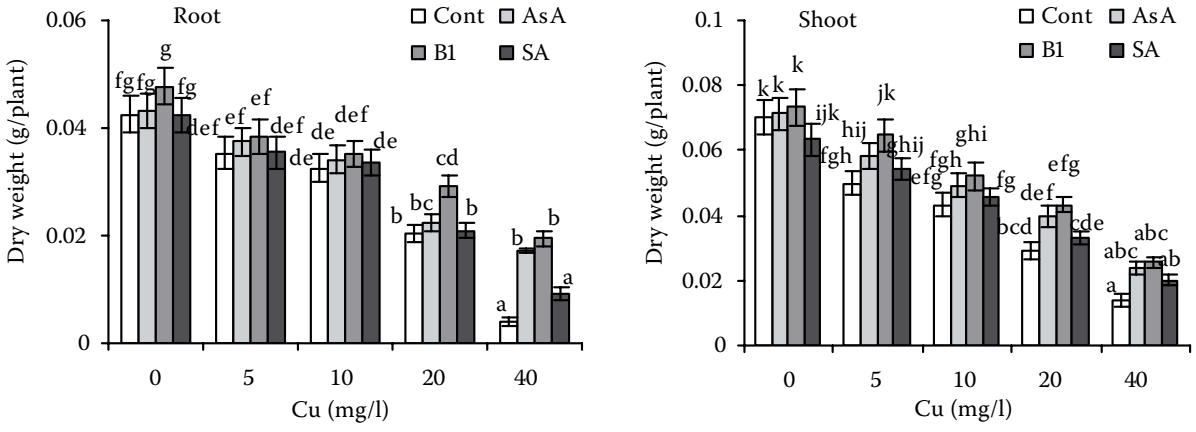
Effect of Cu on growth

The effect of different levels of Cu on the dry matter yield of test wheat plants is presented in Figure 1. The increasing Cu level from 5 mg/l to 40 mg/l reduced dry matter gain in the roots and shoots of test plants. The adverse effects of Cu were markedly demonstrated by wheat plants treated with 40 mg/l Cu. In most cases the pretreatment with AsA, B_1 or SA resulted in a pronounced increase in the production of dry matter yields in the shoots and roots of Cu stressed wheat plants as compared with those of untreated plants (Figure 1).

Effect of Cu on cell wall components

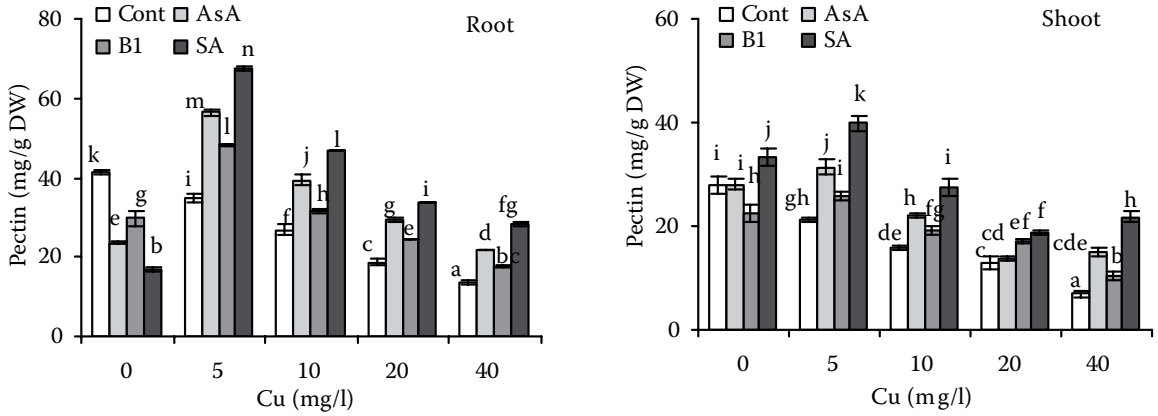
Modification of cell wall components plays an important role in the cell expansion. In this study, pectin, cellulose, hemicellulose and lignin were determined in Cu-stressed wheat plants (Figures 2–5). The content of pectin and cellulose in roots and shoots was significantly lowered with the rise of Cu concentration in the rooting medium. On the other hand, hemicellulose and lignin were raised under all the Cu concentrations.

The adverse effects of Cu treatments on pectin and cellulose contents in roots and shoots were



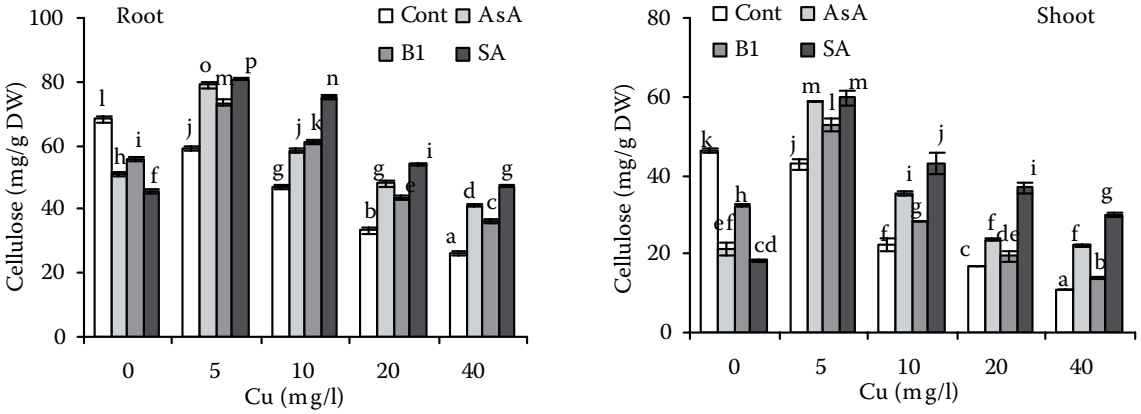
Values are means ± S.E. ($n = 4$); bars carrying different litters are significantly different at $P < 0.05$

Figure 1. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on the dry matter of roots and shoots of wheat plants treated with Cu for 3 days



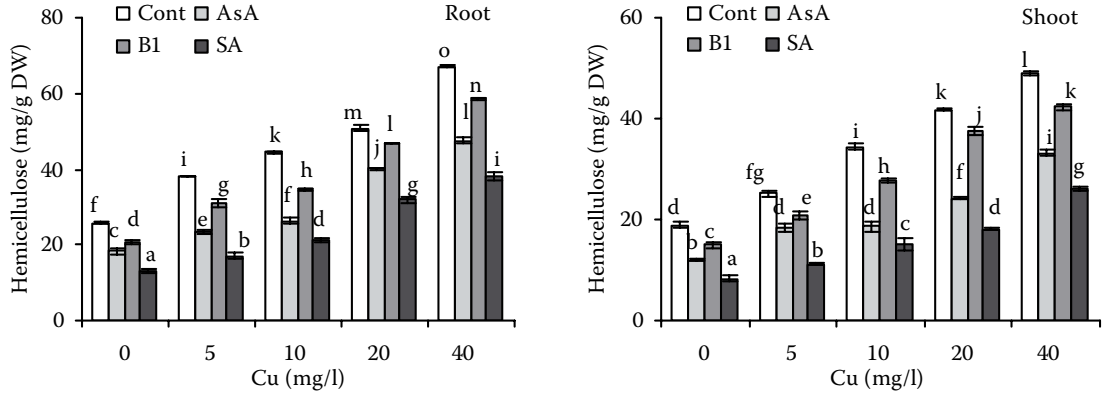
Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 2. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on the pectin of roots and shoots of wheat plants treated with Cu for 3 days



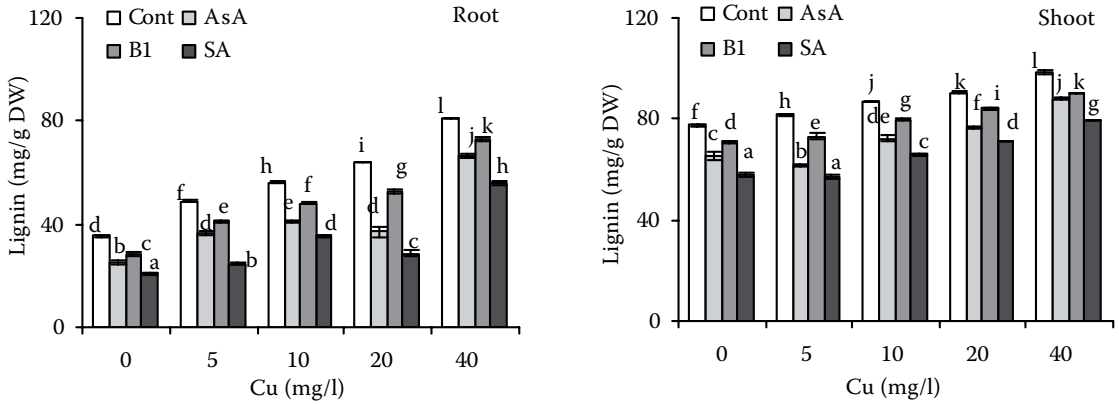
Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 3. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on the cellulose of roots and shoots of wheat plants treated with Cu for 3 days



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 4. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on the hemicellulose of roots and shoots of wheat plants treated with Cu for 3 days



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 5. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on the lignin of roots and shoots of wheat plants treated with Cu for 3 days

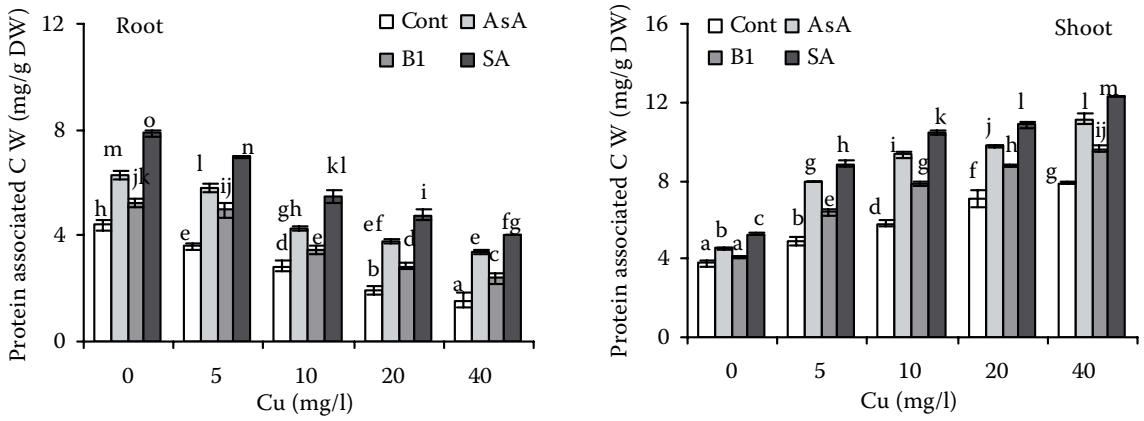
partially or completely alleviated by treatment of seedlings with AsA, B_1 or SA. The applied vitamins or SA were generally effective in partially or completely antagonising the stimulatory effect of Cu stress on hemicellulose and lignin accumulation in roots and shoots of test plants.

Effect of Cu on cell wall associated proteins

In addition to the wall polysaccharides described above, the contents of cell wall associated proteins were estimated since this protein fraction contains enzymes which take part in the wall synthesis and/or degradation (Figure 6). In Cu-stressed plants, cell wall associated proteins were stimulated in shoots, but they significantly decreased in roots. Most of the investigated vitamins or SA with or without Cu had a stimulatory effect on the accumulation of cell wall associated proteins in shoots and roots.

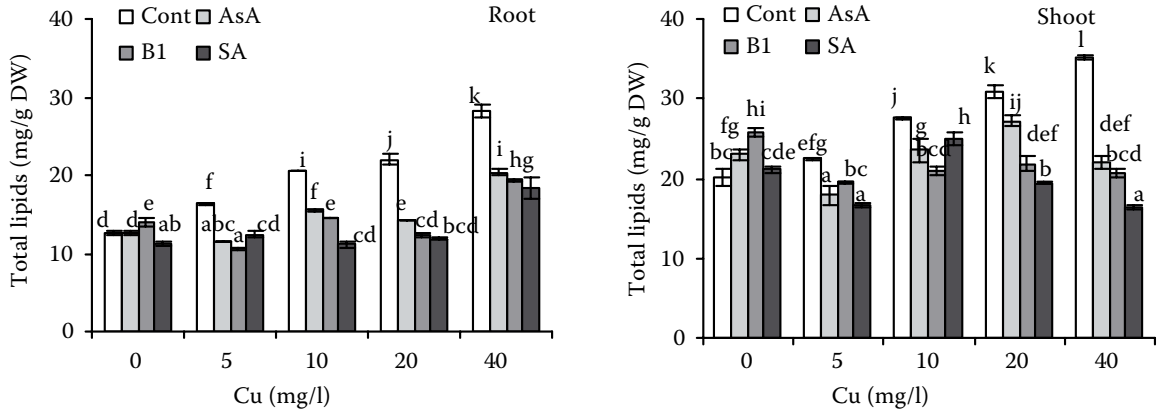
Effect of Cu on lipid fractions

Total lipids and lipid fractions of the studied plants exhibited variable changes in response to the Cu stress and their interaction with the applied vitamins and SA (Figures 7–10). It is clear that all the investigated levels of Cu generally induced a stimulatory effect on the accumulation of total lipids in the roots and shoots of test plants. Treatment with AsA, B_1 or SA had an inhibitory effect on the accumulation of total lipids in the different organs under various concentrations of Cu.



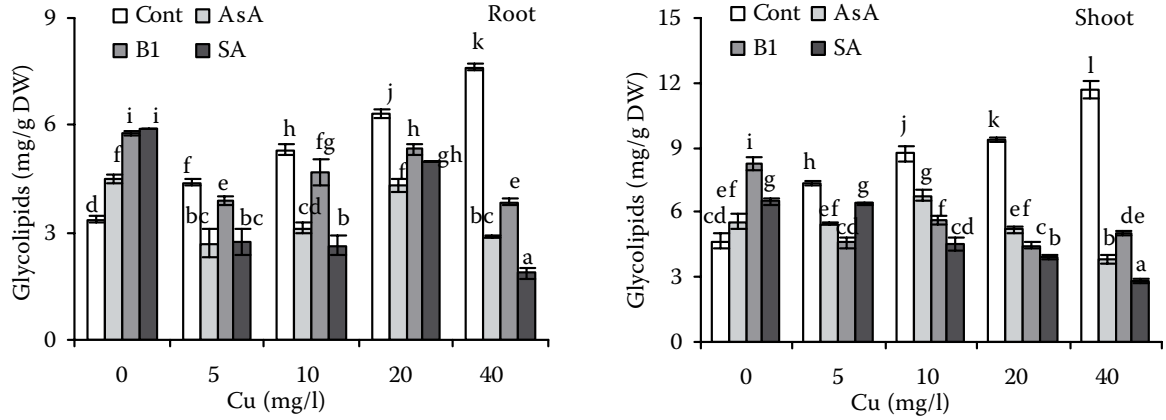
Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 6. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on the cell wall associated proteins of roots and shoots of wheat plants treated with Cu for 3 days



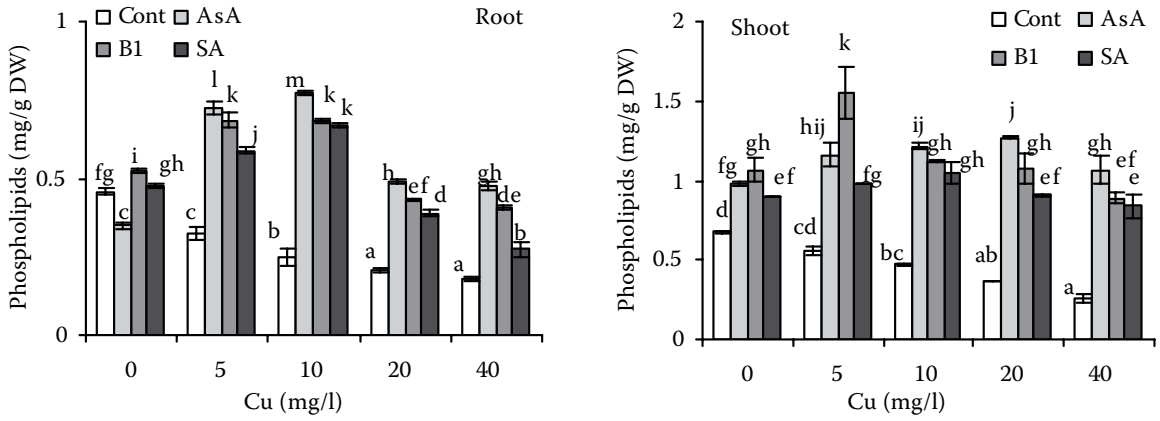
Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 7. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on total lipids of roots and shoots of wheat plants treated with Cu for 3 days



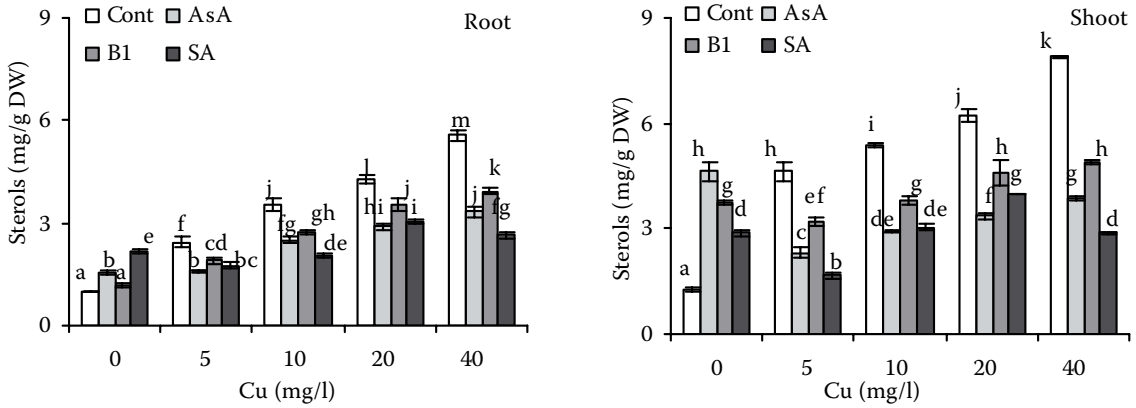
Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 8. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on glycolipids of roots and shoots of wheat plants treated with Cu for 3 days



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

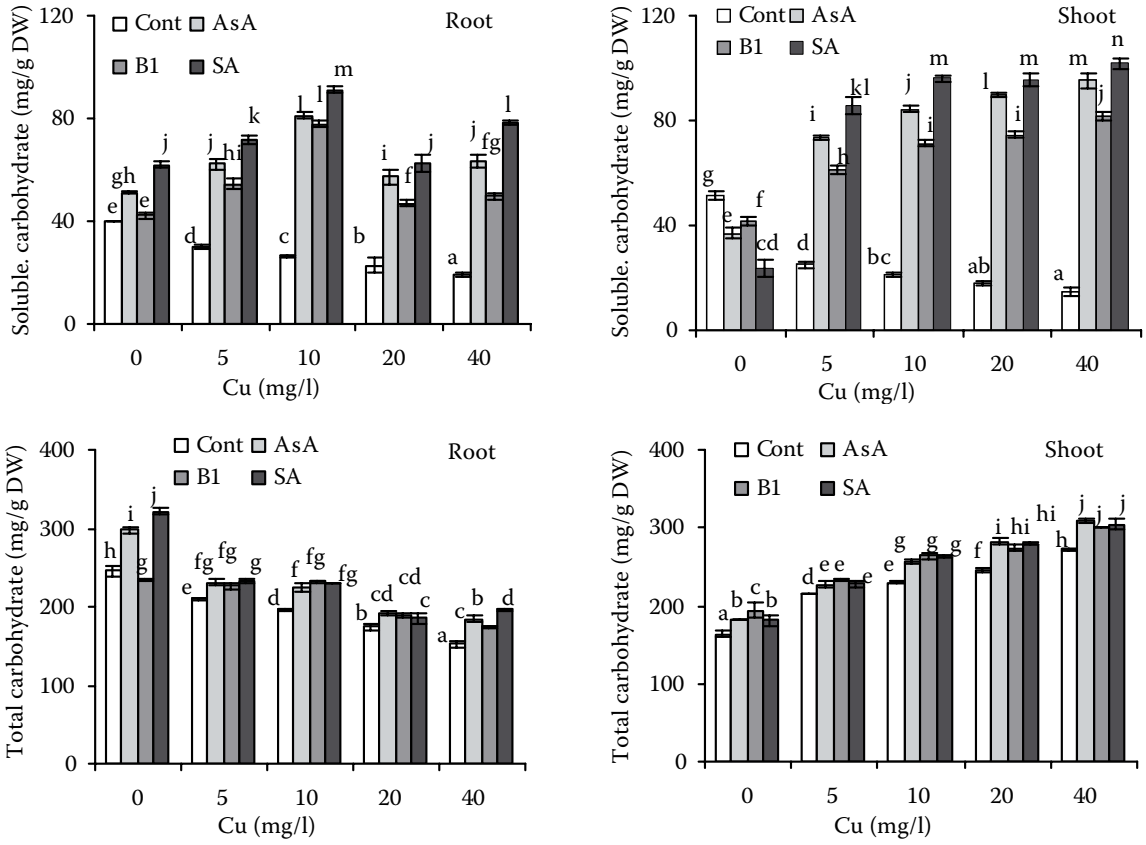
Figure 9. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on phospholipids of roots and shoots of wheat plants treated with Cu for 3 days



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

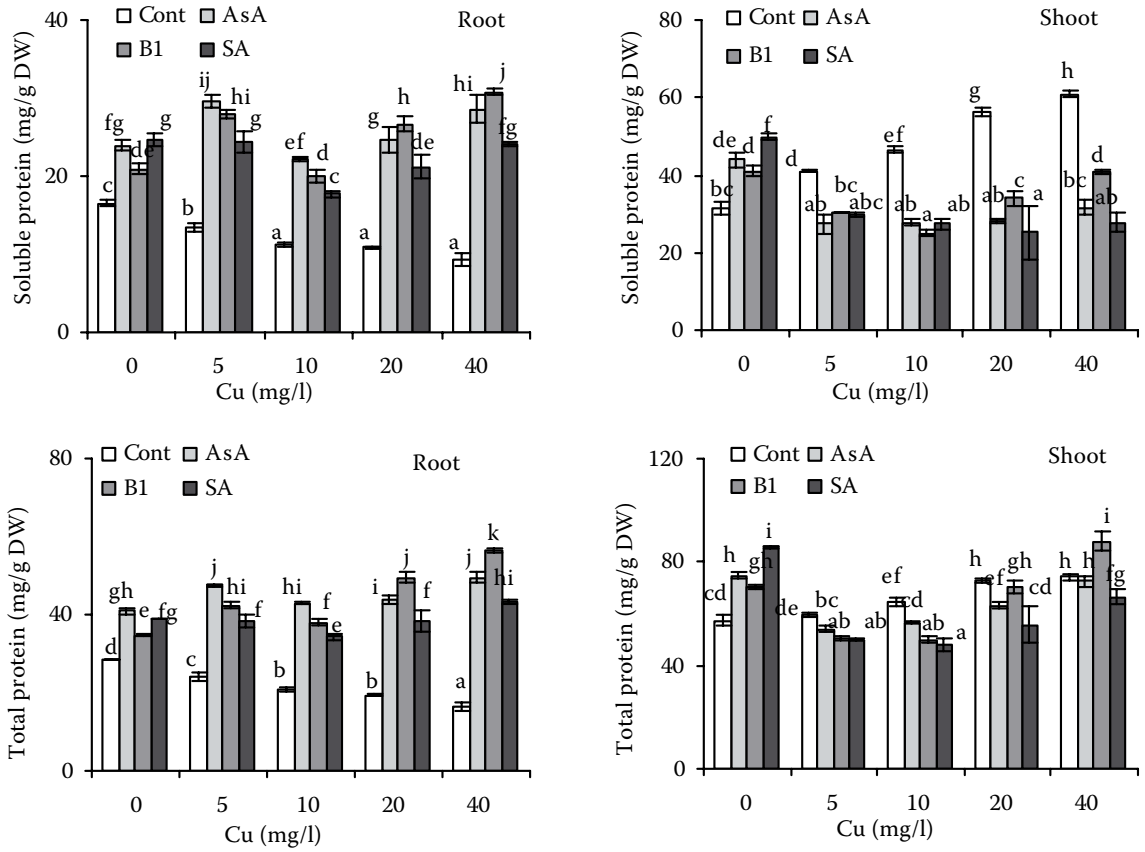
Figure 10. Effect of treatment with 100 ppm ascorbic acid (AsA), thiamine (B1) or sodium salicylate (SA) on sterols of roots and shoots of wheat plants treated with Cu for 3 days

Cu stress significantly increased the glycolipid and sterol fraction of all organs of test plants, while the phospholipid fraction decreased in the roots and shoots of Cu-stressed plants. The applied vitamins or SA were generally effective in partially or completely antagonizing the stimulatory effect of Cu stress on glycolipid and sterol accumulation in the roots and shoots of test plants. On the other hand, phospholipid accumulation in roots and shoots was stimulated by the application of vitamins or SA at all Cu concentrations.



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 11. Effect of different concentrations of Cu on soluble and total carbohydrates (mg/g DW) in roots and shoots of 15-day-old wheat plants treated with Cu for 3 days



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 12. Effect of different concentrations of Cu on soluble and total proteins (mg/g DW) in roots and shoots of 15-day-old wheat plants treated with Cu for 3 days

Effect of Cu on carbohydrate biosynthesis

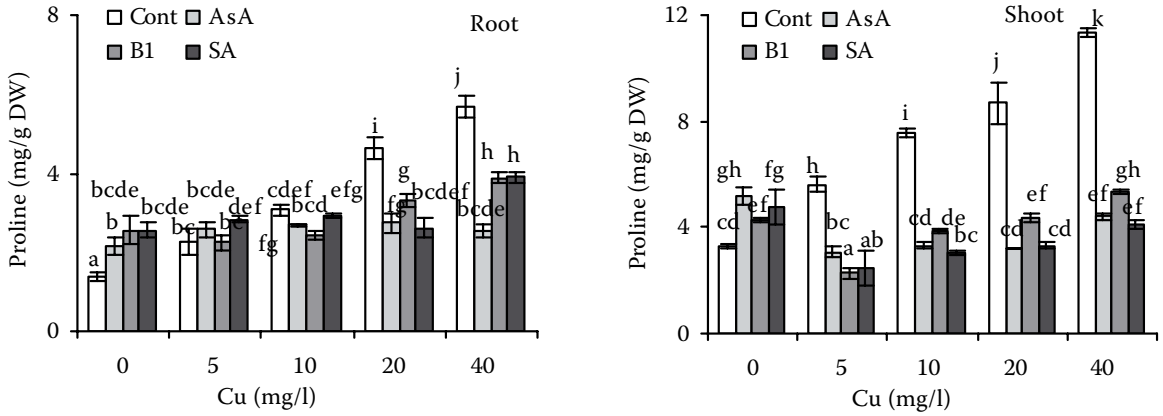
Variations in the production of soluble and total carbohydrates in test wheat plants as affected by Cu supply are presented in Figure 11. It is clear that the investigated levels of Cu generally induced an inhibitory effect on the accumulation of soluble and total carbohydrates in the roots and shoots of wheat plants, except in shoots where the accumulation of total carbohydrates was stimulated by all levels of Cu. Furthermore, vitamin or SA treatments were capable of acting as activators of the accumulation of soluble and total carbohydrates in the roots and shoots of Cu-stressed plants.

Effect of Cu on protein biosynthesis

Changes in soluble and total protein content in wheat plants are shown in Figure 12, which illustrates that the exposure to different Cu concentrations slightly decreased the soluble and total protein content in wheat roots after 3 days of treatment while the accumulation of soluble and total proteins slightly increased in the shoots of wheat with the increase in Cu level. Treatment of seedlings with AsA, B_1 or SA exhibited a stimulatory effect on the accumulation of soluble and total proteins in wheat roots. In shoots vitamins or SA exerted at most Cu levels an inhibitory effect on the accumulation of soluble and total proteins in wheat plants.

Effect of Cu on proline accumulation

The effect of Cu supply on proline accumulation in the shoots and roots of wheat plants is shown in Figure 13. The data revealed that the increase in Cu level in the culture medium had a significant stimulatory effect on the accumulation of proline in the shoots and roots of test wheat plants. On the other hand, vitamins or SA treatment were significantly efficient to reduce the stimulatory effect of Cu stress on proline accumulation.



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

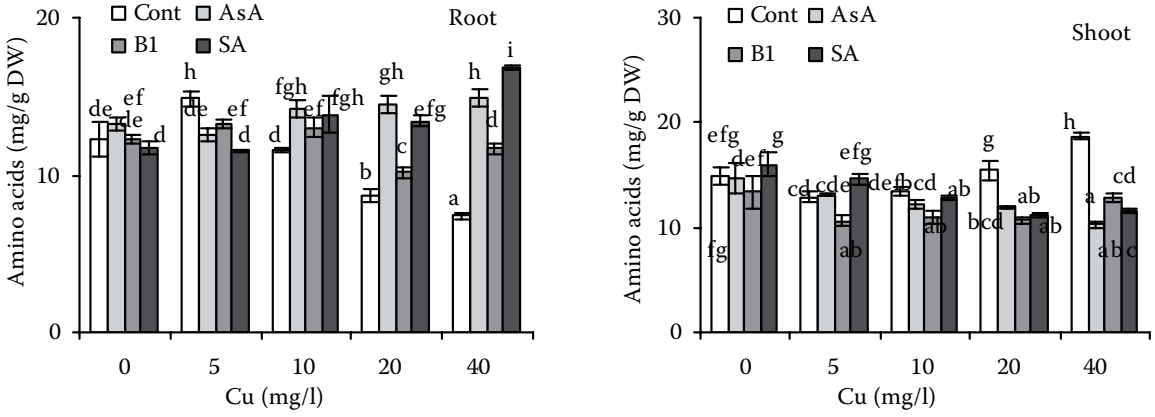
Figure 13. Effect of different concentrations of Cu on proline accumulation (mg/g DW) in roots and shoots of 15-day-old wheat plants treated with Cu for 3 days

Effect of Cu on amino acid biosynthesis

The data presented in Figure 14 clearly demonstrate that the production of other free amino acids in the organs of wheat plants was substantially affected by the various Cu levels with or without vitamin or SA treatments. It is interesting to note that biphasic actions of most of the investigated Cu levels, involving the stimulation of production of other free amino acids in shoots and inhibition of their accumulation in roots were clearly displayed in wheat plants. With respect to vitamin or SA treatment, it is interesting to note that vitamins or SA had a stimulatory effect on the production of other free amino acids in roots and an inhibitory effect on shoots especially at the high levels of Cu (20 mg/l and 40 mg/l).

DISCUSSION

An excess of Cu in soil plays a cytotoxic role, induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis (Lewis *et al*. 2001). The results of this study showed that the increasing Cu levels from 5 mg/l to 40 mg/l reduced dry matter yield in the roots and shoots of test plants. Cu inhibits growth and interferes with important cellular processes such as photosynthesis and respiration (Marschner 1995; Prasad & Strzalka 1999; Yruela 2005). In addition, the exposure of plants to an excess of Cu generates oxidative stress and reactive oxygen species (ROS) (Stadtman & Oliver 1991). Oxidative stress causes disturbance of metabolic pathways



Values are means ± S.E. (n = 4); bars carrying different litters are significantly different at $P < 0.05$

Figure 14. Effect of different concentrations of Cu on other free amino acids biosynthesis (mg/g DW) in roots and shoots of 15-day-old wheat plants treated with Cu for 3 days

and damage to macromolecules (Hegedus *et al*. 2001). In most cases the pretreatment with AsA, B_1 or SA resulted in a pronounced increase in the production of dry matter yields in the shoots and roots of Cu-stressed wheat plants as compared with those of untreated plants. In this context, Chao *et al*. (2010) found out that the pretreatment with AsA or GalL, a biosynthetic precursor of AsA, caused a reduction in Cd toxicity and in Cd-decreased AsA content and AsA/DHA ratio of seedlings. The addition of GalL has also been described to be effective in reducing Cd toxicity of wheat plants (Zhao *et al*. 2005) but it affected other mechanisms of Cd detoxification. Similarly, after the treatment of spring wheat plants with brassinosteroids a decrease in heavy metal content (Cu, Cd, Pb, and Zn) was observed in growth stage 73–75 of the decimal code (DC) (i.e. during the period when the plants are harvested for ensiling purposes (Kroutil *et al*. 2010). Likewise, a decrease in lead content in grains 70–74 in the plants treated at two stages 29–31 DC and 59–60 DC and by 48–70 in the plants of the third group (plants treated at stage 59–60 DC) was determined as compared with the untreated plants. Currently, thiamine can also be added to the list of metabolites utilized by plants to combat oxidative damage (Tunc-Ozdemir *et al*. 2009). On the other hand, Metwally *et al*. (2003) concluded that SA does not alleviate Cd toxicity at the level of antioxidant defence but by affecting other mechanisms of Cd detoxification.

The content of pectin and cellulose in roots and shoots was significantly lowered with the rise of Cu concentration in the rooting medium. On the other hand, hemicellulose and lignin were raised under all the Cu concentrations. This could be important for preventing extension (Van Volkenburgh & Boyer 1985). In addition, in this respect, Wehr *et al*. (2004) concluded that the toxic effect of Cu, Al, and La, especially on root growth, inhibiting the degradation of pectin, which in turns shields hemicellulose, ultimately leads to a reduction in the cell expansion. As one of the possible strategies concurring to avoidance, Colzi *et al*. (2010) suggested that in the tolerant population the presence of Cu in the culture medium triggered the modulation of the cell wall composition decreasing its pectin concentration and increasing the degree of pectin methylation. This response deserves to be deeply investigated in order to evaluate if this change can affect Cu accumulation inside the root and Cu toxicity to the root itself, thus unravelling some physiological strategies concurring to Cu tolerance. It is undoubtedly true that Cu stress retards the plant growth through its influence on the accumulation of lignin, which is considered as one of the constituents of the secondary cell wall. The accumulation of insoluble phenols, such as lignin, in the secondary cell wall was reported in plants exposed to heavy metals and could be associated with an increase in the activity of lignifying peroxidases (Schützendübel *et al*. 2001). Lignification decreases the cell-wall plasticity and, therefore, reduces the cell growth (Schützendübel & Polle 2002). In Cu-treated chamomile, a reduction in root growth was accompanied by brown colouration of this organ, increased peroxidase activity and lignin accumulation (Kováčik & Bačkor 2008; Kováčik & Klejdus 2008). The adverse effects of Cu treatments on pectin and cellulose contents in roots and shoots were partially or completely alleviated by the treatment of seedlings with AsA, B_1 or SA. The applied vitamins or SA were generally effective in partially or completely antagonizing the stimulatory effect of Cu stress on hemicellulose and lignin accumulation in the roots and shoots of test plants. Vitamins or SA could perhaps alleviate the inhibitory effects of Cu stress on glucose incorporation into cell wall polysaccharides. Hamada (2001) concluded that the pretreatment of wheat seedlings with AsA, B_1 or SA could alleviate the inhibitory effects of Cu stress and stimulate growth *via* the enhancement of the photosynthetic rate. Also, Xiong *et al*. (2008) concluded that the higher activities in acid invertases of mine population plants might be associated at least partly with the Cu tolerance of plants, and their higher activities in acid invertases in turn played a role in the maintenance of Cu tolerance by supplying carbon and energy for tolerance mechanisms. Ascorbate is an essential antioxidant in the ascorbate-glutathione pathway, but it also protects enzymes that have prosthetic transition metal ions. Furthermore, it is a cofactor for many enzymes, including those involved in the cell wall synthesis, most notably in the hydroxylation of proline residues (Ishikawa *et al*. 2006). The synthesis of thiamine also responds to environmental conditions. The *Arabidopsis* THI1 gene is induced both during development and by light (Ribeiro *et al*. 2005). Intriguingly, the expression of this gene also responds to stress conditions, including hypoxia, high salinity and sugar deprivation, perhaps because of a need for increased thiamine for respiration or possibly because of a role for thiamine in DNA

repair. Salicylic acid is a potent signalling molecule in plants and is well established to be involved in eliciting specific responses to biotic stresses (Shah 2003). Furthermore, SA is also known to be involved in abiotic stress signalling, including plant responses to heavy metals. The SA pretreatment alleviates Pb- and Hg-induced membrane damage in rice (*Oryza sativa*; Mishra & Choudhuri 1999) and Cd toxicity in barley (*Hordeum vulgare*) and maize (*Zea mays*) seedlings (Pál *et al*. 2002; Metwally *et al*. 2003).

Cell wall associated proteins were stimulated in shoots, but they significantly decreased in the roots of Cu-stressed wheat seedlings. Most of the investigated vitamins or SA with or without Cu had a stimulatory effect on the accumulation of cell wall associated proteins in shoots and roots. Plants may contain specific metal sensors that detect changes in the metal status (deficiency or excess) and trigger signalling cascades that activate the appropriate responses. In higher plants, the involved signal transduction pathways have not been identified yet. Jonak *et al*. (2004) observed that toxic concentrations of Cu activated mitogen-activated protein kinases (MAPKs) in lucerne (*Medicago sativa* L.) seedlings, suggesting that MAPK pathways are activated in response to excess Cu. MAPKs are involved in signal transduction induced by heavy metals and protein phosphorylation events. It remains to establish the extent to what the activation of the respective MAP kinase cascades is metal-dependent or the effect of oxidative stress. On the other hand, the inhibitory effects of Cu on cell wall associated proteins in the roots of test plants support the possibility of a direct effect of the toxic level of Cu on the root system. In this context, Samet *et al*. (1998) found out that Cr^{+} and Cu^{2+} exposure resulted in a relatively small activation of MAPKs. Also, Jouili and El Ferjani (2003) suggested that the reduction of protein amount due to cupric stress could be related to the ability of Cu to interfere with thiol groups of a wide range of enzymes (Fernandes & Henriques 1990) and might therefore produce disorders in protein metabolism. Moreover, the cupric ion is considered as an efficient generator of toxic oxygen species that cause protein degradation (Palma *et al*. 2002).

Cu stress significantly increased total lipids, glycolipids and sterol fraction of all organs of test plants while the phospholipid fraction decreased in the roots and shoots of Cu-stressed plants. The applied vitamins or SA were generally effective in partially or completely antagonising the stimulatory effect of Cu stress on total lipid, glycolipid and sterol accumulation in the roots and shoots of test plants. On the other hand, phospholipid accumulation in roots and shoots was stimulated by the application of vitamins or SA at all Cu concentrations. Heavy-metal stress affects the normal translocation of electrons, resulting in free-radical production that in turn leads to lipid peroxidation (Atal *et al*. 1991). Active oxygen species can damage essential membrane lipids as well as proteins and nucleic acids (Noctor & Foyer 1998). The probable reasons for the increase of glycolipids and sterols and the decrease of phospholipids might be the effect of Cu stress like salinity stress (Wu *et al*. 1998). The alleviation of Cu stress by vitamins and SA might be due to an antioxidant effect of vitamins and SA.

The investigated levels of Cu generally induced an inhibitory effect on the accumulation of soluble and total carbohydrates in the roots and shoots of wheat plants, except the shoots where the total carbohydrates were stimulated by all levels of Cu. Furthermore, vitamins or SA treatments were capable of acting as activators of the accumulation of soluble and total carbohydrates in the roots and shoots of Cu-stressed plants. In this respect, excess Cu may interfere with the biosynthesis of the photosynthetic machinery and may modify the pigment and protein components of photosynthetic membranes (Maksymiec *et al*. 1994). This effect may lead to a decrease in the production of carbohydrates. The results of the previous work and those obtained by other investigators (Maksymiec & Baszyski 1996; Hamada 2001) clearly demonstrate that high applications of Cu inhibit the photosynthetic activity. These results were generally accompanied by reciprocal variations in the dark respiration activity (stimulated). Seedling pretreatment with AsA, B_1 or SA was generally effective in partially or completely antagonizing the inhibitory effects of Cu stress on the net photosynthetic rate (Hamada 2001). It has been reported that Cu can induce a greater chlorophyll decline in non-tolerant plants than in Cu-tolerant plants (Liu & Xiong 2005). This is expected to affect photosynthesis and to reduce the assimilate supply to sink roots, thus inhibiting root growth. Root growth is dependent on the source of carbon and energy supplied by sugars and their development is regulated by sugars (Rolland *et*

al. 2006). Sugars are synthesised in source leaves and translocated to sink roots in higher plants in the form of sucrose to sustain heterotrophic metabolism and growth (Rolland *et al*. 2006). It is believed that acid invertases control and regulate cell division, elongation and differentiation by producing hexoses (glucose and fructose), which not only supply carbon and energy for these events, but also stimulate specific sugar sensors (Rolland *et al*. 2006).

The exposure to different Cu concentrations slightly decreased the content of soluble and total proteins in wheat roots after 3 days of treatment. These results are in accordance with those obtained by Xue-Mei *et al.* (2006) using *Zea mays*, which exhibited a reduction in root length at 200 mg/kg of copper, which was accompanied by a decrease in total soluble protein content. Soluble protein content in organisms, an important indicator of reversible and irreversible changes in metabolism, is known to respond to a wide variety of stressors such as natural and xenobiotic ones (Singh & Tewari 2003). The toxicity of metals can be a result of the generation of ROS that may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death (Bai *et al*. 2003). The inability of *Lemna* fronds to synthesise protein after Cu treatment might be caused by an acute oxidative stress induced by Cu excess in plant cells (Mazhoudi *et al*. 1997). A significant decrease in the protein level under cupric stress could be related to the ability of Cu to interfere with thiol groups of a wide range of enzymes (Fernandes & Henriques 1990) and might therefore produce disorders in protein metabolism. Moreover, the cupric ion is considered as an efficient generator of toxic oxygen species that cause protein degradation (Palma *et al*. 2002). On the other hand, the treatment of seedlings with AsA, B_1 or SA exhibited a stimulatory effect on the accumulation of soluble and total proteins in wheat roots. Maksymiec *et al*. (2007) hypothesised that after a short time exogenous AsA specifically induces the dissipative processes through the reduction of Cu^{2+} to Cu^{+} and, in consequence, it induces the enhancement of radical processes in the chloroplast resulting in a decrease in the potential quantum yield of PSII. Also, thiamine is an essential cofactor in the activity of several enzymes associated with major metabolic pathways, including the Krebs cycle, pentose phosphate pathway, branched-chain amino acid pathway, anaerobic respiration, and pigment biosynthesis. In addition to the important roles of thiamine in plant biosynthetic pathways, limited information suggests that it can also function as an inducer of the accumulation of PR in a salicylic acid-dependent pathway and enhance disease resistance in tobacco, *Arabidopsis*, cucumber and rice (Malamy *et al*. 1996; Ahn *et al*. 2005). In a study with *Chlorella vulgaris*, the most pronounced increases in the content of chlorophylls and proteins at 10^{-4}M SA were observed (Czerpak *et al*. 2002). In terms of the endogenous SA accumulation, heavy metals may increase or decrease its content (Pál *et al*. 2005; Kováčik *et al*. 2009). On the contrary, the accumulation of soluble and total proteins was slightly increased in the shoots of wheat with the increasing Cu level while at most Cu levels vitamins or SA exerted an inhibitory effect on the accumulation of soluble and total proteins in shoots. In this context, Cuypers *et al.* (2002) reported that the application of metals (Cu and Zn) resulted in an increase of protein content in the *Phaseolus vulgaris* primary leaf tissue. A new protein was observed in the primary leaves of *Phaseolus vulgaris* after Cu application, and this protein was identified as a homologue of thylakoid luminal 17.4 kDa proteins from *A. thaliana* (Cuypers *et al*. 2005). The role of these proteins in Cu-induced stress responses is still unknown. It remains to be shown whether any of these proteins can serve as a biomarker of exposure to heavy metals, particularly Cu. It seems that copper excess can influence the plant tissue protein content quite differently, depending on concentration and exposure duration, plant species and genotype specificity, as well as other growth conditions. The remarkable responsiveness in the biosynthesis of protein fractions in the roots and shoots of Cu-stressed wheat plants, which was displayed in the presence of AsA, B_1 or SA treatment, may be taken as a further evidence of the role played by the two applied vitamins or SA and their interaction with Cu stress, in modifying the functional activities at the sites of synthesis, resulting in retarded or enhanced biosynthesis which can lead to alterations for maintaining the integrity of plant adaptation mechanisms.

The increase in Cu level in the culture medium had a significant stimulatory effect on the accumulation of proline in the shoots and roots of test wheat plants. This is in accordance with the alleviating effect of this amino acid under excess of heavy metals (Mehta & Gaur 1999).

On the other hand, vitamin or SA treatment was significantly efficient to reduce the stimulatory effect of Cu stress on proline accumulation. Free proline has been proposed to act as a hydroxyl radical and singlet oxygen scavenger (Smirnoff & Cumbes 1989; Alia Mohanty & Matysik 2001); it also alleviates free-radical damage induced by heavy metals by maintaining a more reducing environment in plant cells (Surasak *et al*. 2002) since proline accumulation could be regarded as an indicator of stress severity (Stewart & Larher 1980). On the other hand, Agarwal and Pandey (2004) suggested that proline protects enzymes and membranes against oxidative stress. In accordance with this, vitamins or SA could alleviate the Cu-stress *via* decreasing reactive oxygen species.

With respect to other free amino acids, biphasic actions of the majority of the investigated Cu levels, involving the stimulation of production of other free amino acids in shoots and inhibition of their accumulation in roots, were clearly displayed in wheat plants. Vitamins or SA had a stimulatory effect on the production of other free amino acids in roots and an inhibitory effect on shoots especially at the high levels of Cu stress. Excessive Cu causes a drastic change in nitrogen metabolism affecting enzymes involved in nitrate reduction and amino acid metabolism and leading to the diminution of total nitrogen (Llorens *et al*. 2000). Do Amarante *et al.* (2005) concluded that the composition of nitrogenous compounds transported in the xylem to the shoot can reflect some changes in root amino acids. The pool of amino acids cycling between the roots and shoots is considered to serve as a signal for the plant internal N status (Aslam *et al*. 2001). Bačkor *et al*. (2007) suggested that endogenous SA stimulates changes of amino acids and this increase may provide protection against further stress impacts e.g. through the synthesis of phytochelatins serving as important chelators of free metal ions also in green algae (Bačkor *et al*., 2007). However, Kováčik *et al*. (2010) concluded that Cu led to a depletion of endogenous SA content in Cu + SA treatment and this depletion had no direct effect on the accumulation of the majority of detected phenolic acids, while individual amino acids seemed to be affected.

We can conclude that Cu stress exerted its adverse effect through increasing the lignin content and decreasing phospholipids. On the other hand, AsA, B1 or SA could alleviate the adverse effect of Cu stress through increasing the content of phospholipids and decreasing the content of lignin.

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