The protective effects of cobalt on potato seedling leaves during osmotic stress

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Abstract. The protective effects of cobalt on potato seedling leaves during osmotic stress were reported and discussed in this paper. The results showed that the membrane damage was significantly alleviated after 24 h of osmotic stress when cobalt of appropriate concentrations was added in PEG solutions. During -1.0 MPa PEG osmotic stress cobalt of 25 µmol L-1 inhibited ethylene production rate significantly. Cobalt of 25 µmol L-1 had no significant influence on the content of thiobarbituric acid reacting substances or chlorophyll in the first 16 h. When the stress lasted 20 h and 24 h, treatment with cobalt effectively restrained the increment in the levels of reactive oxygen species, hampered the decline in the content of putrescine, spermidine and spermine. The decline in the activities of anti-oxidative enzymes, thus inhibited the accumulation of thiobarbituric acid reacting substances and alleviated the reduction of chlorophyll content. It can be concluded that when the potato leaves were deeply stressed and damaged, through the inhibition of ethylene production, cobalt alleviated the decline in polyamine content and the activities of anti-oxidative enzymes, and hence alleviated the increment in reactive oxygen species levels and membrane damage and showed protective effects on the leaves.

Keywords: Cobalt; Ethylene; Osmotic stress; Polyamines; Potato.

Abbreviations: ACC, 1-aminocyclopropane 1-carboxylic acid; Co, cobalt; ETH, ethylene; MSI, membrane stability index; PAS, Polyamines; Put, putrescine; Spd, spermidine; Spm, spermine; ROS, reactive oxygen species; TBARS, thiobarbituric acid reacting substances.

Introduction

Cobalt (Co) is a kind of trace element and heavy metal found in soil (Hansen et al., 2001; Guevara et al., 2002) that can be incorporated into the active site of urease and render the enzyme inactive (Yamaguchi et al., 1999). Co concentration may be higher in serpentine, acidic, calcareous, or peaty soils, and may enter soils owing to the pollution of metal refineries and vehicular and aircraft exhausts (Freedman and Hutchinson, 1981). Excess Co induces yield reduction and an inhibition in assimilate production in leaves, and even inhibits the export of photoassimilates to roots and other sinks (Rauser and Samarakoon, 1980). Excess Co also causes oxidative stresses (Tewari et al., 2002) and may result in phytotoxicity to plants (Chatterjee and Chatterjee, 2003). However, cobalt is unequivocally essential for leguminous crops as it is required for nitrogen fixation by bacteria in root nodules (Witte et al., 2002), and it even has beneficial effects on some non-leguminous crops (Locke et al., 2000).

Co is an inhibitor of 1-aminocyclopropane 1-carboxylic acid (ACC) oxidase and does inhibit ethylene (ETH) production (Lau and Yang, 1976; Locke et al., 2000). ETH shares the common precursor S-adenosylmethionine with spermidine (Spd) and spermine (Spm), so ETH and polyamines (Spd and Spm) are generally regarded to compete for a limited pool of S-adenosylmethionine (Wang et al., 2002). Furthermore, ETH may promote the oxidation of polyamines through its influence on reactive oxygen species (ROS) levels when osmotic stress became aggravated (Li et al., 2004). Since Co can reduce ETH production, we can hypothesize that it may have some influence on the content of polyamines, and even on membrane damage during environmental stress. However, to the best of our knowledge, little research has been conducted in this field, and the physiological effects of Co in proper concentration on the stressed plants remain unclear. Whether and how Co exerts its influence on cell membranes during environmental stresses needs to be investigated.

Materials and Methods

Plant Culture and Treatments

Potato (Solanum tuberosum L. cv. Gannongshu NO. 1) internodal segments were cultured in Murashige and Skoog (MS) medium which contained 30 g L-1 sucrose. The growth conditions were 22°C, 16-h photoperiod, and 120
µmol m−2 s−1 light intensity. After 30 days of culture the fully expanded leaves were picked and immersed in distilled water. PEG (PEG-4000) solutions, cobalt chloride solutions, or PEG (PEG-4000) solution added with cobalt chloride to the final concentration of 25 µmol L−1. The temperature was 22°C, and the illumination was 120 µmol m−2 s−1. After a certain amount of treatment the leaves were thoroughly rinsed with distilled water and used for the following measurements.

**Membrane Stability Index (MSI) and the Contents of Thiobarbituric Acid Reacting Substances (TBARS) and Chlorophyll**

The membrane stability index (MSI) was determined according to the method of Sairam et al. (1997/1998). TBARS content was measured following the method of Dhindsa and Matowe (1981). The content was calculated using its extinction coefficient of 155 mmol L−1 cm−1. Chlorophyll was extracted by homogenizing 0.3 g fresh weight of the leaves in 10 ml 100% methanol in the morning. After centrifugation for 10 min at 500 rpm, the chlorophyll in the supernatant was measured spectrophotometrically, and the chlorophyll content of the leaves was calculated according to the method of Lichtenthaler (1984).

**ETH Production Rate**

ETH production rate was analyzed following the procedure described in our previous work (Li et al., 2004). Gas chromatography (Model GC-9A, Altex-Beckman Inc., Japan) with a column (Paropark) at a column temperature of 90°C was used. N2 was used as flow-gas.

**Polyamine Content**

The extraction of polyamine and HPLC analysis were conducted according to the method of Flores and Galston (1982). A programmable liquid chromatography (Model Waters 600E, Waters Inc., USA) was used in the measurement of the concentrations of these amines. The solvent system consisted of methanol : distilled water (65% methanol) at a flow rate of 1 mL min−1. The benzoylated extracts were eluted at room temperature through a reverse-phase column (Waters Symmetry C18, 3.9 mm × 150 mm, 5 µm in particle size) at 254 nm with a UV detector.

**Reactive Oxygen Species (ROS) Levels**

The production rate of H2O2 was determined following the method of Manuel et al. (1986). The production rate of O2− was measured using the method in our previous work (Li et al., 2004).

**Anti-oxidative Enzyme Essays**

Superoxide dismutase (SOD) activity was determined spectrophotometrically according to the method of Spirochalla and Desborough (1990). One unit of SOD was defined as the amount of enzyme required to inhibit ferricytochrome C reduction by 50%. Activities of catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (POD) were measured following the methods described by Lin and Wang (2002). CAT activity was determined following the consumption of H2O2 (extinction coefficient 39.4 mmol L−1 cm−1) at 240 nm for 2 min. APX activity was determined following the decrease in A290 for 3 min (extinction coefficient 2.8 mmol L−1 cm−1), corrections were made for oxidation of ascorbate in the absence of H2O2. POD activity was based on the determination of guaiacol oxidation (extinction coefficient 26.6 mmol L−1 cm−1) at 470 nm by H2O2.

**Statistical Analysis**

All experiments were performed at least three times and each value was presented as mean + or - standard error (S.E.). The data were statistically analyzed by one-way ANOVA using SPSS statistical software (SPSS for Windows, Release 10) to evaluate whether the means were significantly different, taking \( P < 0.05 \) as significant.

**Results**

**Effects of Different Concentrations of Cobalt on Membrane Stability Index (MSI) and ETH Production Under Osmotic Stress**

The membrane stability index (MSI) of the leaves showed no significant changes after 24 h of immersion in the cobalt solutions, the concentration of which increased from 0 to 125 µmol L−1. However, when the concentrations of cobalt rose to 600 or 3000 µmol L−1, MSI of the leaves decreased markedly (Figure 1).

![Figure 1. Influence of different concentrations of cobalt on membrane stability index (MSI) (%) in potato seedling leaves after 24 h of osmotic stresses. Vertical lines in each point show + S.E. (n = 4) \( P < 0.05 \).](image-url)
ETH production rate in the leaves eased downward as cobalt concentrations in distilled water or in -1.0 MPa PEG solution gradually increased after 24 h of treatments (Figure 2).

**Effects of 25 µmol L⁻¹ Cobalt on Contents of TBARS and Chlorophyll**

The contents of TBARS and chlorophyll showed no significant changes as the osmotic stress was initiated, but as the stress continued, TBARS content increased while chlorophyll content fell noticeably (Figure 3A-B). Treatment with cobalt had no significant influence on the content of TBARS and chlorophyll initially, but when the stress lasted 20 h and 24 h, cobalt significantly alleviated the increment in TBARS content and the decline in chlorophyll content (P < 0.05) (Figure 3A-B).

**Effects of 25 µmol L⁻¹ Cobalt on ETH Production Rate and Polyamine Contents**

ETH production rate varied markedly with the process of osmotic stress: it fell as the osmotic stress lasted from 0 to 8 h, then increased gradually (Figure 4A). Polyamines, spermidine (Spd) and spermine (Spm), together with their precursor putrescine (Put), were the three major amines in potato seedling leaves. Put content peaked when the stress lasted 8 h. Spd and Spm contents reached their peaks when the stress lasted 4 h, and then their contents fell gradually (Figure 4B-D). Cobalt of 25 µmol L⁻¹ did not alter the changing pattern of either ETH production rate or polyamine contents during the osmotic stress, but it reduced ETH production and promoted Put content during the whole course of the osmotic stress (P < 0.05), and somewhat promoted the content of Spd and Spm when the stress lasted 20 h and 24 h (P < 0.05) (Figure 4A-D).

**Effects of 25 µmol L⁻¹ Cobalt on the Activities of Anti-Oxidative Enzymes During Osmotic Stress**

The activities of SOD, CAT and APX increased initially during osmotic stress, then decreased gradually as the stress was prolonged (Figure 5A-C). POD activity showed negligible changes at first, and afterward decreased markedly (Figure 5D). Cobalt treatment had no significant influence on the activities of SOD, CAT, APX or POD in the first 16 h of stress (P > 0.05), but after that cobalt treatment significantly alleviated the decrease in the activities of these anti-oxidative enzymes (P < 0.05) (Figure 5A-D).

**Effects of 25 µmol L⁻¹ Cobalt on H₂O₂ and O₂⁻ Production Rate**

The production rate of H₂O₂ and O₂⁻ showed no significant changes as the stress began, and then it increased significantly (Figure 6A-B). Treatment with 25 µmol L⁻¹ cobalt chloride had no significant influence on the changing pattern in the production rate of H₂O₂ and O₂⁻ caused by osmotic stress at first (P > 0.05), but when the stress lasted...
Figure 4. Influence of cobalt on ethylene production rate (pmol g\(^{-1}\) DW h\(^{-1}\)) and polyamines content (nmol g\(^{-1}\) DW) in potato seedling leaves during osmotic stress. Osmotic stress: -1.0 MPa PEG solution; Osmotic stress + cobalt: -1.0 MPa PEG solution added with 25 µmol L\(^{-1}\) cobalt chloride. Vertical lines in each point show + or - S.E. (n = 3) (P < 0.05).

Figure 5. Influence of cobalt on the activities of SOD (10\(^3\) Units g\(^{-1}\) DW), CAT (10\(^5\) U g\(^{-1}\) DW min\(^{-1}\)), APX (µmol g\(^{-1}\) DW min\(^{-1}\)) and POD (mmol g\(^{-1}\) DW min\(^{-1}\)) in potato seedling leaves during osmotic stress. Osmotic stress: -1.0 MPa PEG solution; Osmotic stress + cobalt: -1.0 MPa PEG solution added with 25 µmol L\(^{-1}\) cobalt chloride. Vertical lines in each point show + or - S.E. (n = 3) (P < 0.05).
20 h and 24 h, treatment with cobalt significantly alleviated the increment in the production of H$_2$O$_2$ and O$_2^-$ ($P<0.05$) (Figure 6A-B).

**Discussion**

Cobalt has many physiological effects on plant growth and development, and some of these are harmful, such as reducing the activity of urease (Yamaguchi et al., 1999; Witte et al., 2002) and hampering the transportation of assimilates in excess concentration (Rauser and Samarakoon, 1980). On the other hand, some of the effects are beneficial for high plants. For example, Co promotes the growth of seedlings and alleviates the senescence of aged tissues as it inhibits the activities of ACC oxidase and reduced ETH production (Lau and Yang, 1976). Our results also proved that Co inhibited ETH production in potato seedling leaves during PEG osmotic stresses (Figure 2 and 4A).

Co reduced the production of ETH involved in plant stress physiology in many aspects, but few reports about whether, and how, Co exerts its influences on plant during environmental stresses can be found, and the physiological roles of Co in stressed plants remain unclear.

ETH is regulated by internal signals during plant development and in response to environmental stresses, such as wounding, hypoxia, ozone, or freezing (Wang et al., 2002). ETH is a potent modulator of plant growth and development, and even stress physiology. For instance, ETH influences the metabolism of reactive oxygen species, polyamines, and even the activities of anti-oxidative enzymes during stresses (Pandey et al., 2000; Wang et al., 2002). Polyamines are implicated in a wide range of biological processes (Evans and Malmberg, 1989; Martin-Tanguy, 2001). A close correlation between ETH and polyamines has been demonstrated in many cases (Katoh et al., 1987; Botha and Whitehead, 1992), and in this paper it was proved that the inhibition in ETH production alleviated the decrease in polyamine content as the stress was prolonged (Figure 4A-D). The results were consistent with our previous work in deeply stressed wheat and Glycyrrhiza seedlings (Li et al., 2004; Li and Wang, 2004). Polyamines are low molecular mass polycations. When they are bound to the membrane target, they exert protective effects on cell membrane when the plants are under stress (Pandey et al., 2000). Through alleviating the reduction in polyamine content, Co may exert some beneficial effects on cell membrane.

ROS are produced naturally during cell metabolism in photosynthesis, photorespiration, fatty acid oxidation, senescence, and other processes (Turpaev, 2002). Generally, ROS concentrations are low, but during senescence or intense stresses, ROS accumulation and their concentrations may be above a certain “threshold” (Turpaev, 2002). In the other way, plants are endowed mechanisms to deal with ROS. For example, anti-oxidative enzymes and antioxidants can reduce ROS levels (Carlos et al., 1999). According to Chae and Lee (2001), ETH promoted superoxide production in carrot cells during carbon starvation. Our results also showed that when the stress lasted 20 h and 24 h, Co significantly alleviated the decrease in the activities of SOD, CAT, POD and APX (Figure 5A-D) through an inhibition in the increment of ETH production (Figures 2 and 4A).

Treatment with Co alleviated the reduction in polyamine contents and in the activities of anti-oxidative enzymes when osmotic stress was prolonged (Figures 4B-D, 5A-D). These changes may influence ROS levels, and our results demonstrate that the increment in the production rate of H$_2$O$_2$ and O$_2^-$ caused by osmotic stress was restrained significantly by Co treatment when the stress lasted 20 h and 24 h (Figure 6A-B). As the stress continued, lipid peroxidation and chlorophyll damage in the leaves of potato seedlings became aggravated (Figure 3A-B). Furthermore, treatment with cobalt alleviated the lipid peroxidation, the reduction in chlorophyll content, and cell membrane damage (Figures 1 and 3A-B). These results showed that cobalt alleviated leaf damage as the osmotic stress was aggravated.
From the above results, it can be concluded that when leaves were not deeply stressed, although Co inhibited ETH production significantly, it had no significant influence on ROS levels or lipid peroxidation. When the leaves were deeply stressed and damaged, through inhibiting ETH production, Co alleviated the decline in polyamine contents and in the activities of anti-oxidative enzymes. In this way, Co restrained the increment in ROS levels and lipid peroxidation degrees and thus showed protective effects.

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Literature Cited


鈷對滲透脅迫下馬鈴薯幼苗葉片的保護作用

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本文通過Co對滲透脅迫下馬鈴薯幼苗葉片乙烯產生、活性氧水準、多胺含量、抗氧化酶活性及細胞膜穩定指數的影響，研究報導了Co對滲透脅迫下馬鈴薯幼苗葉片的保護作用及機制。研究結果表明，-1.0 MPa PEG溶液滲透脅迫24小時，低濃度的Co減輕脅迫所致的細胞膜損傷。在-1.0 MPa PEG溶液滲透脅迫過程中氧化鈷顯著抑制了乙烯的產生。在滲透脅迫的初始階段，細胞內活性氧及脂質過氧化水準無顯著增加，25 µmol L⁻¹氧化鈷對此也無顯著影響。隨著脅迫時間的延長，25 µmol L⁻¹氧化鈷顯著減輕了腐胺、精胺、亞精胺含量以及抗氧化酶活性的下降，因此有效地減輕了活性氧含量、脂質過氧化程度的增加，以及葉綠素含量和細胞膜穩定指數的下降。因此，可以認為Co通過抑制乙烯產生，相對提高多胺含量及抗氧化酶活性，抑制了活性氧含量的提高、葉綠素的降解及細胞膜的損傷，從而對滲透脅迫下馬鈴薯幼苗的葉片產生一定的保護作用。

關鍵詞：鈷；乙烯；滲透脅迫；多胺；馬鈴薯。