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CoCl$_2$ Aminoethoxyvinylglycine   AVG

Vicia faba   CoCl$_2$ AVG

12 h   20 μmol L^{-1} CoCl$_2$ SOD
CAT APX   20 μmol L^{-1} AVG   20 μmol L^{-1} CoCl$_2$ 20 μmol L^{-1}
AVG   Put   Spd   Spm   ROS
45 ℃   12 h   20 μmol L^{-1} CoCl$_2$ 20 μmol L^{-1} AVG

3

CoCl$_2$ AVG

THE PROTECTIVE EFFECTS OF ETHYLENE PRODUCTION INHIBITORS ON *VICIA FABA* SEEDLING LEAVES UNDER HEAT STRESS

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Abstract   Here   we studied the effects of ethylene production inhibitors   cobalt chloride   CoCl$_2$ and aminoethoxyvinylglycine   AVG   on the leaves of *Vicia faba* seedlings under heat stress. Ethylene production in the leaves was inhibited significantly before heat stress and after 12 h heat stress when the seedlings were irrigated with 20 μmol L^{-1} CoCl$_2$ or 20 μmol L^{-1} AVG solution for 7 days. Treatment with 20 μmol L^{-1} CoCl$_2$ increased significantly the activities of SOD   CAT and APX before heat stress   while the treatment with 20 μmol L^{-1} AVG had no significant influences. Treatments with 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} AVG did not influence the contents of putrescine   Put   spermidine   Spd   spermine   Spm   chlorophyll and the production rate of reactive oxygen species   ROS . However   after 12 h heat stress of 45 ℃   treatments significantly promoted the above values. These results showed that when plants suffered 12 h heat stress of 45 ℃ treatment with 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} AVG inhibited ethylene production   increased the content of these amines and enhanced the activities of the anti-oxidative enzymes   restrained the enhancement in the content of ROS   and alleviated the reduction in membrane stability index   by which the leaves of *Vicia faba* seedlings were protected.

Key words   Aminoethoxyvinylglycine   Cobalt   Ethylene   Heat stress   *Vicia faba*

Cobalt   Co   a kind of trace element and heavy metal that existed comprehensively in soil   Hansen *et al*. 2001   Guevara *et al*. 2002   is essential for animals and micro-organisms as a constitutional part of vita-

min B_{12} or as an essential component for N_2-fixation and rhizobial growth Samecka-Cymerman & Kempers 2001 Marschner 1995 . However as Co can enter soil owing to the pollution of metal refineries vehicular and aircraft exhausts Freedman & Hutchinson 1981 Abraham & Hunt 1995 most researches focused on the adverse effects of excess Co on plants e.g. excess Co caused oxidative stress Chatterjee & Chatterjee 2003 Tewari *et al*. 2002 plant chlorosis Kleizaite *et al*. 2004 inhibition in assimilate production and the export of photoassimilates from leaves to roots and other sinks Rauser & Samarakoon 1980 and the constant influence on the ecosystems Abraham & Hunt 1995 . Only a little information is available on the requirement of cobalt by higher plants such as symbiotic N_2-fixation by leguminous plants Marschner 1995 .

It was attested that Co had significant influences on reactive oxygen species ROS levels and even the activities of anti-oxidative enzymes Tewari *et al*. 2002 Bisht & Mehrotra 1989 . In the other way Co is an inhibitor of 1-aminocyclopropane 1-carboxylic acid ACC oxidase and can inhibit ethylene production predominantly Lau & Yang 1976 Locke *et al*. 2000 . Aminoethoxyvinylglycine AVG an inhibitor of ACC synthase can inhibit the production of ethylene Aziz *et al*. 1997 Even-Chen *et al*. 1982 . As ROS and ethylene do play important roles in plant membrane systems under environmental stsesses Co and AVG may exert their influences on plant stress physiology through their direct influences on the metabolism of ROS and ethylene. However to our knowledge little information can be found in this field and the role of Co in plant stress physiology do need further research.

1 Materials and Methods

1.1 Plant culture and treatment

Vicia faba seeds were surfacely sterilized with $HClO_4$ 8% active Cl_2 for 5 min rinsed with distilled water then immersed in distilled water for 3 days the water was replaced every 12 h . The seeds were germinated in a growth chamber in dark and at a temperature of 25 ± 1 ℃ for 48 h then germinated seeds were transferred to plastic pots filled with vermiculite cultured in a growth chamber at a temperature of 25 ± 1 ℃ with a 16-h photoperiod and a photon flux density of 150 μmol m^{-2} s^{-1} watered with Hoagland solution Haogland & Arnon 1957 every day. After 30 days of culture the seedlings were watered with Hoagland solution served as control or Hoagland solution supplemented with cobalt

chloride $CoCl_2$ or AVG of different concentrations 1 4 20 100 500 or 2 500 μmol L^{-1} for 7 days and after that duration Co or AVG had been accumulated in certain concentrations in the leaves respectively. Then the seedlings were transferred into a growth chamber with a temperature of 45 ± 1 ℃ and a photon flux density of 150 μmol m^{-2} s^{-1} for heat treatment. After 12 h heat stress the leaves withered slightly the second and third pairs of leaves counting from the base of the plants were picked from the seedlings for the following measurements.

1.2 Measurement of membrane stability index MSI and thiobarbituric acid reacting substance content

MSI was determined following the method of our previous work Li *et al*. 2004b . Thiobarbituric acid reacting substance TBARS content was measured according to the method of Dhindsa and Matowe 1981 .

1.3 Measurement of chlorophyll content

Chlorophyll was extracted by homogenizing 0.5 g fresh weight of the leaves in 10 ml 100% methanol. After centrifugation for 10 min at 500 r min^{-1} the chlorophyll in the supernatant was measured spectrophotometrically and chlorophyll content of the leaves was calculated using the method of Lichtenthaler 1984 .

1.4 Measurement of ethylene production

Ethylene production was measured following the procedure of Li *et al*. 2004a . Gas-chromatography Model GC-9A Altex-Beckman Inc. Japan with a column Paropark at a column temperature of 90 ℃ was used N_2 was used as flow-gas.

1.5 Measurement of polyamine content

Polyamine extraction and HPLC analysis were carried out according to the method of Flores and Galston 1982 . Plant extracts and authentic standards of putrescine Put spermidine Spd and spermine Spm were benzoylated following the procedure described by Flores and Galston 1982 . The concentration of these polyamines was measured using programmable liquid chromatography Model Waters 600E Waters Inc. USA . The solvent system consisted of methanol 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. The temperature of the column was 25 ℃ the results were quantified with a C-R 25 integrator.

1.6 Measurement of reactive oxygen levels

The production rate of H_2O_2 was measured according to the method of Manuel *et al*. 1986 . The production rate of O_2^- was determined following the method in our

previous work Li *et al* . 2004a .

1.7   Measurement of the activities of anti-oxidative enzymes

Superoxide dismutase SOD activity was determined spectrophotometrically following the method of Spychalla and Desborough 1990 . One unit of SOD is defined as the amount of enzyme that inhibits the rate of ferricytochrome C reduction by 50% . Activities of catalase CAT and ascorbate peroxidase APX were measured according to the methods of Lin and Wang 2002 . CAT activity was determined according to the consumption of H_2O_2 extinction coefficient 39.4 mmol L^{-1} cm^{-1} at 240 nm in 2 min. APX activity was determined according to the decrease in A_{290} for 3 min extinction coefficient 2.8 mmol L^{-1} cm^{-1} corrections were made for oxidation of ascorbate in the absence of H_2O_2 Lin & Wang 2002 .

1.8   Statistical analysis

All experiments were performed three or more times and each value was presented as mean ± standard error *SE* . The data were statistically analyzed by One-Way ANOVA using SPSS10 statistical software taking $p <$ 0.05 as significant.

2   Results

2.1   Influences of different concentrations of $CoCl_2$ and AVG on the MSI

MSI of the leaves of *Vicia faba* seedling showed no significant changes with the treatment of $CoCl_2$ solutions of 1 4 20 and 100 μmol L^{-1} before the heat stress $p >$ 0.05 whereas declined significantly when the concentration of $CoCl_2$ increased to 500 and 2 500 μmol L^{-1} p < 0.05 . After 12 h heat stress of 45 ℃ treatment with $CoCl_2$ solutions of the concentrations mentioned above all alleviated the decreases in MSI significantly $p < 0.05$ Fig. 1a .

Treatment with different concentrations of AVG solutions had no significant influences on MSI of the seedlings leaves before the heat stress $p > 0.05$ but after 12 h heat stress AVG alleviated the decreases in MSI of the leaves markedly $p < 0.05$ Fig. 1b .

2.2   Influences of 20 μmol L^{-1} $CoCl_2$ and 20 μmol L^{-1} AVG on ethylene production and polyamine content

Ethylene production of the leaves was inhibited predominantly $p < 0.05$ but the content of Put Spd and Spm was not significantly influenced with the treatment of 20 μmol L^{-1} $CoCl_2$ and 20 μmol L^{-1} AVG before the heat stress $p > 0.05$ Fig. 2a – d . Both ethylene production and polyamine content in the control irrigated

with Hoagland solution only increased markedly after 12 h heat stress $p < 0.05$ and the content of these amines was improved while the production of ethylene was inhibited significantly in the leaves of the seedlings that irrigated with the $CoCl_2$ and AVG solutions $p < 0.05$ Fig. 2a – d .

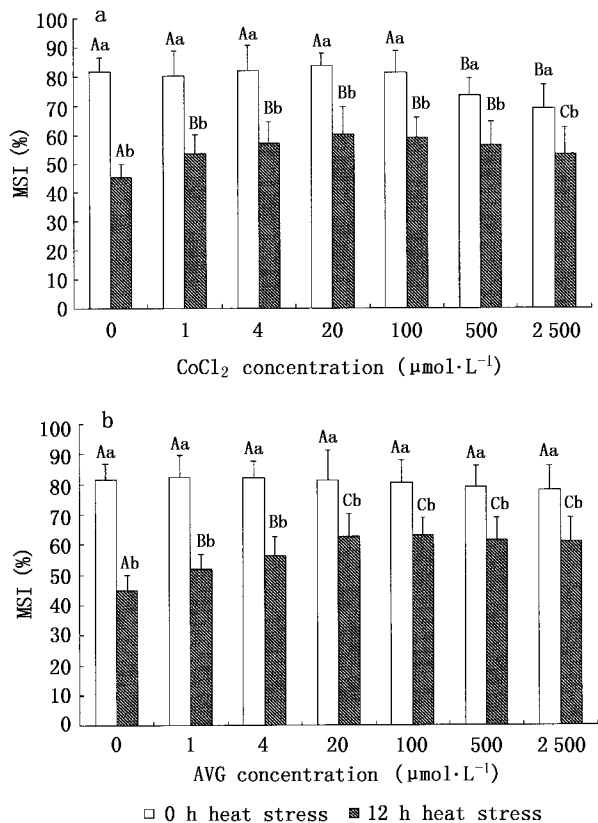Fig.1   Influences of different concentrations of $CoCl_2$ and aminoethoxyvinylglycine AVG on the membrane stability index MSI of *Vicia faba* seedlings leaves after 0 and 12 h heat stress of 45 ℃

Different capital letters above the bars indicate significant differences p < 0.05 of different treatments before or after heat stress different small letters indicate significant differences $p < 0.05$ of the same treatment before and after heat stress

2.3   Influences of 20 μmol L^{-1} $CoCl_2$ and 20 μmol L^{-1} AVG on TBARS and chlorophyll′ s content

TBARS content in the leaves increased while chlorophyll content decreased significantly after 12 h heat stress in the control Fig. 3a – b . Before the heat stress treatment with $CoCl_2$ and AVG had no significant differences with the control in the content of TBARS and chlorophyll $p > 0.05$ but when the heat stress lasted 12 h the increase in TBARS content and the decrease in chlorophyll content were restrained effectively by the treatment of 20 μmol L^{-1} $CoCl_2$ and 20 μmol L^{-1} AVG $p < 0.05$ Fig. 3a – b .
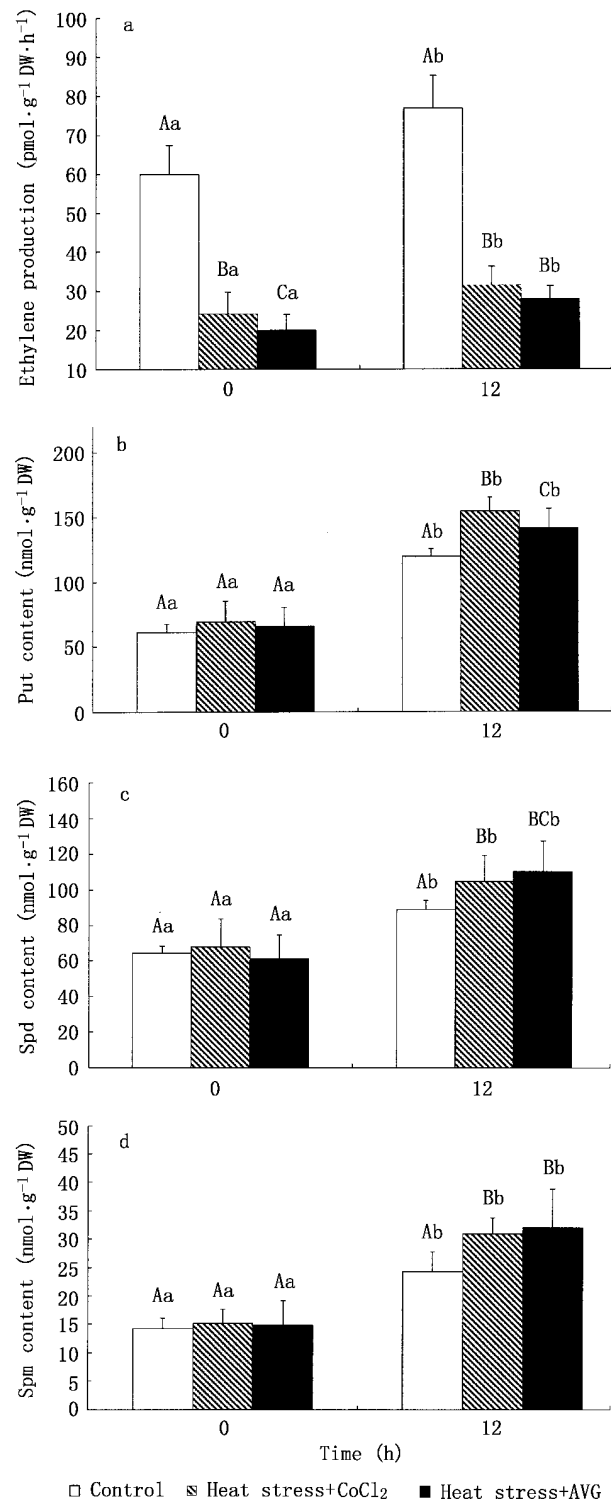
Fig.2 Influences of 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} aminoethoxyvin-
ylglycine AVG on ethylene production and content of putrescine
Put spermidine Spd and spermine Spm in the leaves of
Vicia faba seedlings after 0 and 12 h heat stress of 45 ℃
Different capital letters above the bars indicate significant differences p
< 0.05 of different treatments before or after heat stress different small let-
ters indicate significant differences $p < 0.05$ of the same treatments before
and after heat stress

2.4 Influences of 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} AVG on the production rate of H$_2$O$_2$ and O$_2^-$

20 μmol L^{-1} CoCl$_2$ improved while 20 μmol L^{-1} AVG inhibited the production rate of H$_2$O$_2$ and O$_2^-$ not significantly before the heat stress $p > 0.05$.

However when the heat stress lasted 12 h the increment in the production rate of H$_2$O$_2$ and O$_2^-$ caused by heat stress was significantly restrained with the treatment of CoCl$_2$ and AVG $p < 0.05$ Fig. 4a – b .
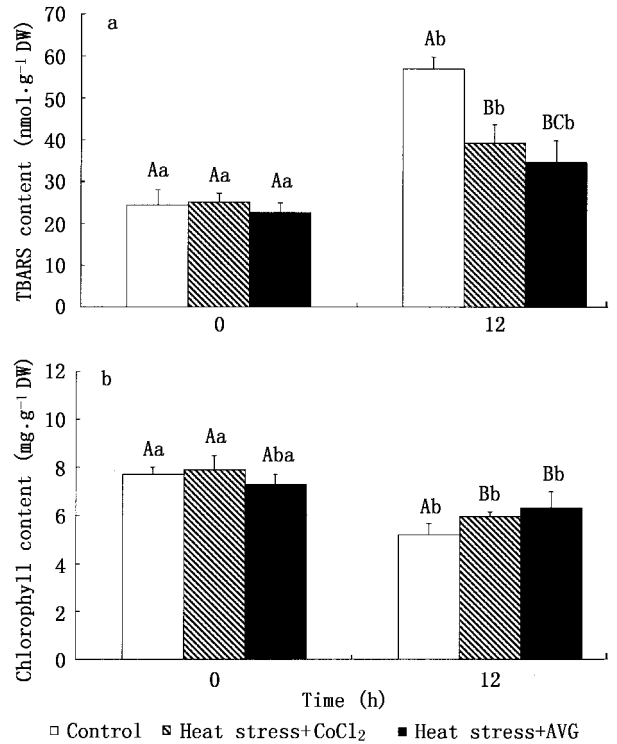

Fig.3 Influences of 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} aminoethoxyvin-
ylglycine AVG on the content of thiobarbituric acid reacting
substances TBARS and chlorophyll in the leaves of *Vicia faba*
seedlings after 0 and 12 h heat stress of 45 ℃
Different capital letters above the bars indicate significant differences p
< 0.05 of different treatments before or after heat stress different small let-
ters indicate significant differences $p < 0.05$ of the same treatments before
and after heat stress

2.5 Influences of 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} AVG on the activities of anti-oxidative enzymes

The activities of SOD CAT and APX were significantly improved with the treatment of 20 μmol L^{-1} CoCl$_2$ before the heat stress while the activities of the three anti-oxidative enzymes were not significantly influenced with the treatment of 20 μmol L^{-1} AVG. After 12 h heat stress the activities of the three anti-oxidative enzymes in control decreased evidently and the activities of the anti-oxidative enzymes in the leaves of the *Vicia faba* seedlings treated with CoCl$_2$ and AVG were significantly higher than the control Fig. 5a – c .
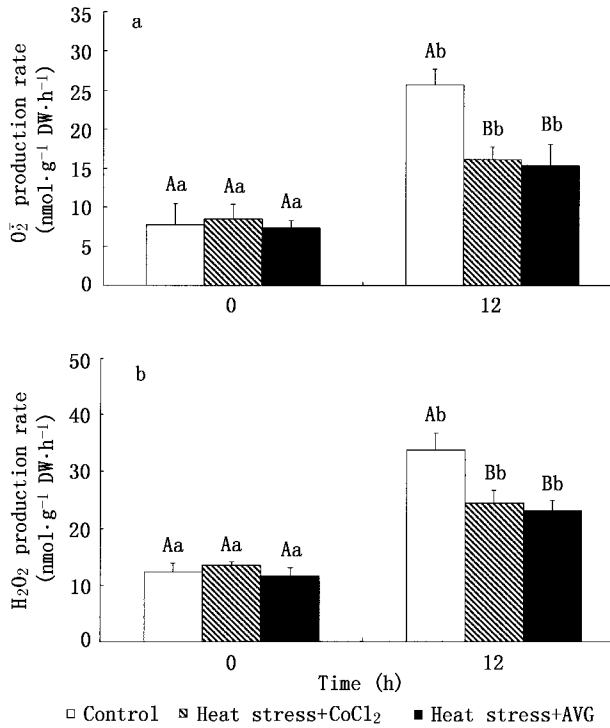
Fig.4   Influences of 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} aminoethoxyvin-
ylglycine AVG on the production rate of H$_2$O$_2$ and O$_2^-$ in the leaves
of *Vicia faba* seedlings after 0 and 12 h heat stress of 45 ℃
Different capital letters above the bars indicate significant differences p
< 0.05 of different treatments before or after heat stress different small let-
ters indicate significant differences $p < 0.05$ of the same treatments before
and after heat stress

3   Discussion

Plants grow over a wide range of temperatures apart
from encountering large seasonal variations in tempera-
ture plants also face temperature variations of tens of de-
grees centigrade in a short time   thus induce heat stresses
in plants Gaspar *et al* . 2002 . Heat stresses are asso-
ciated intimately with the metabolic processes in plants
and may induce plants undergo oxidative stresses caused
by a rapid accumulation of ROS and even membrane dam-
age Becana *et al* . 1998 Gaspar *et al* . 2002 . Our
work also attested that the accumulation of ROS Fig. 4a
– b and even membrane damage caused by 12 h heat
stress Fig. 1a – b . In the other way the plants are en-
dowed with an impressive array of antioxidative enzymes
and antioxidants to cope with the ROS Dhindsa & Ma-
towe 1981 Manuel *et al* . 1986 . According to Tewari
et al . 2002   Co treatment significantly increased the
activities of SOD and APX our work also attested CoCl$_2$
treatment significantly improved the activities of SOD
CAT and APX Fig. 5a – c . The improvement in the
activities of the anti-oxidative enzymes may be quite bene-
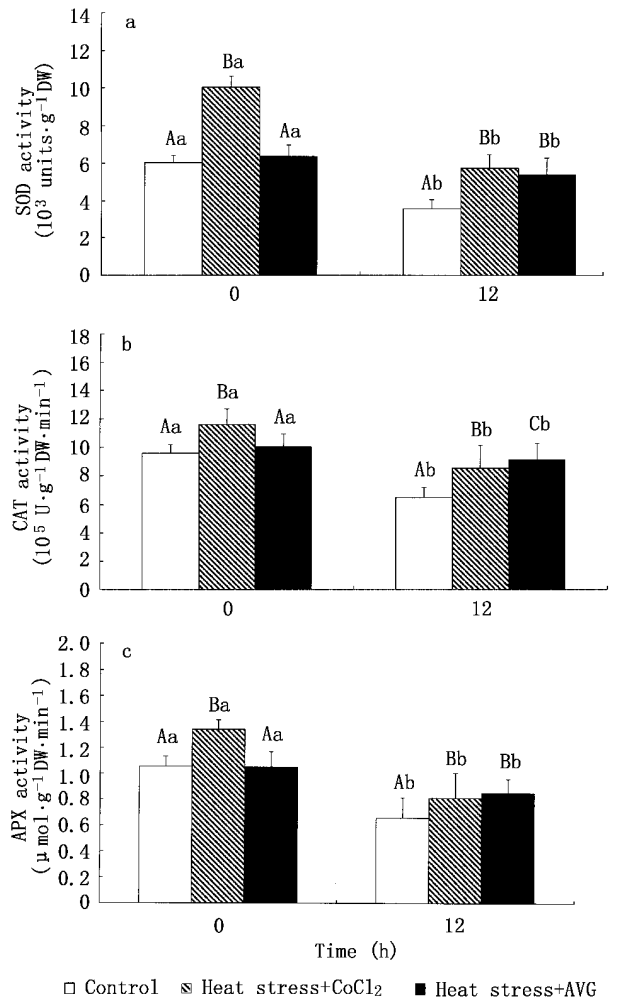ficial for the plants to deal with the increment in ROS

Fig.5   Influences of 20 μmol L^{-1} CoCl$_2$ and 20 μmol L^{-1} aminoethoxyvin-
ylglycine AVG on the activities of superoxide dismutase SOD
catalase CAT and ascorbate peroxidase APX in the leaves of
Vicia faba seedlings after 0 and 12 h heat stress of 45 ℃
Different capital letters above the bars indicate significant differences p
< 0.05 of different treatments before or after heat stress different small let-
ters indicate significant differences $p < 0.05$ of the same treatments before
and after heat stress

induced by heat stress.

Ethylene is an important plant growth substance in
plant metabolic network under stresses and has a close
correlation with lipid peroxidation and membrane perme-
ability Johnson & Ecker 1998 Saftner & Bald
1990 . The two important polyamines Spd and Spm use
the common precursor S-adenosylmethionine SAM with
ethylene so it was generally regarded that polyamines
Spd and Spm and ethylene compete for the available
SAM when the pool of SAM was not large enough for the
synthesis of Spd Spm and ethylene Martin-Tanguy
2001 Pandey *et al* . 2000 . In our previous paper it
was proved that when wheat seedlings leaves are under ag-
gravated osmotic stress and cell membrane damage ethy-
lene promoted polyamines oxidation and thus reduced

polyamine content　Li *et al*．　2004a．In this paper it
was also attested that through inhibit ethylene production
polyamines content was relatively improved markedly after
12 h heat stress　Fig．2a－d．

　　Polyamines are low molecular mass polycations exist-
ed ubiquitously in plant kingdom and implicate in a wide
range of biological processes　Bouchereau *et al*．　1999
Martin-Tanguy　2001．At cellular pH value polyamines
behave as cations and can be bound to membrane targets
to exert their protective effects　Mizrahi *et al*．　1989
Tadolini *et al*．　1984　It was also reported that
polyamines could improve the activities of anti-oxidative
enzymes and thus enhanced its ability in abolishing ROS
　Li *et al*．　2004a　2004b　Xu *et al*．　2001．There-
fore　polyamines generally exert the protective effects
when plants are under environmental stresses　Galston ＆
Sawhney　1990　Young ＆ Galston　1983．

　　Excess concentrations of Co in the growth medium
produced visual symptoms of toxicity that intensified with
increasing level and duration of Co supply　Tewari *et
al*．　2002．However　when the concentration was low
there were not the above adverse effects　Tewari *et al*．
2002．Our results also showed high concentrations of Co
reduced the membrane stability index　Fig．1a　but
when Co were in proper concentrations　it showed protec-
tive effects on the leaves as it alleviated the increment in
the accumulation of TBARS　Fig．3a　the reduction in
MSI　Figs．1a　and chlorophyll content　Fig．3b　after
12 h heat stress．

　　The results showed that when the plants suffered 12
h heat stress of 45 ℃　treatment with 20 μmol· L^{-1}
cobalt and 20 μmol L^{-1} AVG inhibited ethylene produc-
tion　improved the content of these amines and enhanced
the activities of the anti-oxidative enzymes relatively　re-
strained the enhancement of ROS content　and alleviated
the reduction in MSI　thus showed the protective effects
on the leaves of *Vicia faba* seedlings．

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