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The Combined Effect of 2,4-Epibrassinolide and Chilling Stress on Tomato Cultivars Differing in Maturity

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Abstract

Low temperature (but above-freezing) during germination and early seedling growth of chilling-sensitive crop is one of the most significant limiting factors in the productivity. 2,4-Epibrassinolide (24-EB) is one of the most active forms of brassinosteroids are multifunctional plant hormones that can regulate development and respond to abiotic stresses. The effect of seed-pretreatment with 24-EB (12.5 μg/L) on photosynthetic characteristics, membrane permeability, lipid peroxidation and antioxidant activities under chilling stress were investigated in tomato (*Lycopersicon esculentum Mill.*) cultivars: Kulon (early ripening) and Yakhont (mid-early). Results showed that the use of 24-EB led to more pronounced changes in the pigment composition in Yakhont in the absence of a stress factor, whereas in Kulon under chilling stress (2 °C for 24 h). 24-EB pretreatment minimized the damage to cell membranes in tomato plants caused by chilling stress. The tolerance to chilling stress in Yakhont was higher than Kulon (by electrolyte leakage and content of malondialdehyde assay). Under these conditions, oxidative processes in plants of Yakhont did not show significant difference. We have not established the effect of 24-EB on the level of low molecular weight antioxidants in tomato cultivars (measured by inhibition of 2,2-diphenyl-1-picrylhydrazyl free radical method). The antioxidant activity of leaf extracts in Yakhont was twice as high as in Kulon under all experimental conditions (with/without 24-EB, 22/2 °C). It was concluded that the less pronounced reaction of plants of Yakhont to the use of 24-EB and chilling stress is due to their genetically determined higher cold resistance than that of Kulon.

Keywords: *Solanum Lycopersicum*, 2,2-Diphenyl-1-Picrylhydrazyl, 2,4-Epibrassinolide, Electrolyte Leakage, Lipid Peroxidation, Photosynthetic Pigments

List of Abbreviations:

24-EB: 2,4-Epibrassinolide,
DPPH: 2,2-diphenyl-1-picrylhydrazyl,
ROS: Reactive Oxygen Species,
MDA: Malondialdehyde,
AOA: Antioxidant Activity,
LHC: Light Harvesting Complex

Introduction

Low temperature (but above-freezing) during early period of growth can be detrimental to subsequent productivity of agricultural crops [1]. Tomato (*Lycopersicon esculentum Mill.*), a subtropical plant, is an important worldwide consumed crop. Being a chilling-sensitive plant, tomato grows best at temperature range of 16-29 °C with minimum of 11 °C during night [2]. Chilling stress can cause lipid cell membrane destruction, increased reactive oxygen species (ROS), malondialdehyde (MDA) content, and increased relative conductivity [3]. One of the ways to reduce the damaging effect of low temperature on plants is the use of growth regulators. Many plants growth regulators are synthetic analogs of endogenous phytohormones [4].

Brassinosteroids are widely used class of natural steroidal plant hormones. Through the signal transduction pathway, brassinosteroids interact with a variety of transcription factors via a series of phosphorylation cascades to regulate the expression of target genes (including stress-responsive genes) [5]. 2,4-Epibrassinolide (24-EB) is a biologically active compound of the brassinosteroids that to act on growth and development of plants [4,6]. Many studies have shown that exogenous 24-EB could confer resistance to agricultural crops against various environmental factors (low and high temperature, drought, salinity, heavy metal toxicity) [6-8]. However, the physiological functions of brassinosteroids in plants are not yet fully understood. When assessing the effectiveness of the action of 24-EB on plants, some authors noted a different reaction of cultivars, especially when exposed to unfavorable environmental factors. For example, the cultivar specificity of the 24-EB effect was revealed when determining the activities of antioxidant enzymes in wheat under salt stress [9]. 24-EB increased the activity of antioxidant enzymes in tomato plants, while cold-resistant cultivars had higher activities than non-resistant ones [10]. In winter wheat seedlings, the endogenous level of 24-EB depended on the degree of frost resistance of the cultivar [11].

Keeping in mind the anti-stress properties of brassinosteroids, the purpose of this study was to evaluate the effects of 24-EB on photosynthetic characteristics, antioxidant activities and cold resistance (measured by membrane permeability and lipid peroxidation) in two cultivars of tomato differing in maturity. In this study, we were the first to investigate the effects of 24-EB on genetically determined level of cold resistance tomato cultivars.

Material and Methods

Plant Material and Experimental Conditions

Seeds of the contrasting tomato (*L. esculentum Mill.*) cultivars-Kulon (early ripening) and Yakhont (medium early) were obtained from the Voronezh vegetable experimental station (Russia). Tomato seeds soaked in an aqueous solution of Epin®-Extra (commercial formulation), containing 24-EB (12.5 μg/L) for 2 h. Seeds imbibed with distilled water were considered as the control. Controls and seeds with 24-EB were germinated in the soil at temperature 22 °C, photoperiod 16/8 h (day/night) with an illumination intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 21 days (Figure 1). Then the tomato plants were divided into two groups. One group of the plant was cooled in the dark at 2 °C for 24 h. The second group of the plant was kept at 22 °C for 24 h in the dark.

Figure 1: Tomato plants at the age of 21 days. Plants grown on a soil substrate at a temperature of 22°C, photoperiod 16/8 h (day/night) with an illumination intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Electrolyte Leakage Assay

The membrane permeability in tomato leaves was measured by the electrolyte leakage from their leaf disks into distilled water on electrical conductivity meter SG7-ELK (Mettler Toledo, Switzerland) [12].

Determination of Lipid Peroxidation

Lipid peroxidation was determined as the content of MDA – an indicator of the degree of plant oxidative stress, using the 2-thiobarbituric acid reaction [13].

Determination of Antioxidant Activity (AOA)

AOA of the extracts from leaves were analyzed by investigating their ability to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical in an ethanol solution [14]. The percentage of DPPH inhibition was calculated by Lo et al. [15] formula.

Determination of Photosynthetic Pigments Content

The allocation of photosynthetic pigments (chlorophylls a (Chl a), chlorophylls b (Chl b) and total carotenoids (car)) were used acetone (80 %). Concentrations of pigments were estimated by Wellburn [16] equations.

Determination of The Quantity of Chlorophylls in The Light Harvesting Complex (LHC)

The quantity of chlorophylls in LHC was calculated on the basis that the entire amount of Chl b was found in the LHC, and that the ratio of Chl a/Chl b in LHC was equal to 1.2 [17].

Determination of The Dry Matter Content in The Leaves

The dry matter content in the tomato leaves was determined after drying in oven at 105 °C to a constant weight and expressed as a percentage of the initial fresh weight of the sample.

Data Analysis

T-test software (ISI Institute Inc., USA) was used for data analysis. Data were reported as mean ± SE values of triplicate experiments. Student's t-test of unpaired samples at P ≤ 0.05 was used. Origin 6.1 software (Origin Lab, USA) was used to draw charts.

Results and Discussion

Effect of Chilling Stress on Membranes Permeability

One of the markers of the chilling injuries of plants is damage to their membranes in the form of electrolyte leakage [7]. 24-EB reduces the negative effect of chilling stress on membranes of plants [6,10]. According to the data, at 22 °C, there were no differences between the cultivars in the output of electrolytes from the leaves (Table 1). The chilling stress (2 °C) induced a significant increase in the leakage of ions through membranes both the cultivars. EL in leaves increased with over time (1, 3 and 6 h) chilling stress. It is established that that the output of electrolytes from the cells of Yakhont it was lower than that of Kulon. Pre-sowing treatment of seeds with 24-EB significantly reduced the release of electrolytes from the leaves of Yakhont plants, especially at chilling stress. The chilling stress (2 °C for 24 h) triggered the maximum leakage of the ions both the cultivars, especially, in the control plants. Therefore, this chilling stress mode was used by us in subsequent experiments.

Variants	**22 °C**	**Immediately after chilling stress (2 °C for 1-24 h)**			
		1	**3**	**6**	**24**
cv.Kulon					
Control	10.05±0.90^{a}	16.31±1.61^{c}	15.20±1.14^{c}	17.64±1.32^{c}	29.31±2.85^{e}
24-EB	8.22±0.64^{b}	13.42±0.66^{d}	14.65±1.05^{dc}	14.58±1.46^{c}	26.25±2.46^{f}
cv.Yakhont					
Control	7.43±0.86^{b}	12.56±1.24^{d}	16.22±1.62^{e}	16.71±1.67^{e}	21.54±1.14^{f}
24-EB	8.84±0.61^{b}	10.63±1.37^{d}	12.02±1.31^{d}	15.62±1.54^{e}	17.48±1.29^{eg}

Table 1: The effects of seed-pretreatment with 24-epibrassinolide (24-EB) on the electrolyte leakage from leaves of tomato plants after variable periods of chilling stress (2 °C), % of total electrolyte efflux Mean values ± SE, Different lowercase letters (a, b, c …) indicate significant differences between treatments (P ≤ 0.05) according to Student's t-test of unpaired samples

Effect of Chilling Stress on Dry Matter Content in the Leaves

In both tomato cultivars, 24-EB did not affect the dry matter content in the leaves, but after chilling stress (2 °C for 24 h), this parameter increased in all variants (Table 2). This indicated a decrease in the content of free water in their tissues under chilling stress.

Parameters	Tomato cultivar	Experimental conditions			
		22 °C		2 °C for 24 h	
		Control	24-EB	Control	24-EB
Chl a	Kulon	10.57±0.47[a]	9.85±0.78[a]	9.91±0.82[a]	13.78±0.83[b]
	Yakhont	9.93±1.17[a]	12.16±0.86[b]	12.80±0.52[b]	14.59±0.93[c]
Chl b	Kulon	3.70±0.31[d]	3.84±0.21[d]	3.26±0.13[d]	5.04±0.47[e]
	Yakhont	3.38±0.32[d]	4.94±0.57[e]	4.36±0.07[e]	5.04±0.82[e]
Total Chl	Kulon	14.27[f]	13.69[f]	13.17[f]	18.82[g]
	Yakhont	13.31[f]	17.10[g]	17.16[g]	19.67[g]
Carotenoids	Kulon	2.43±0.07[h]	2.12±0.09[h]	2.25±0.23[h]	3.31±0.24[i]
	Yakhont	2.21±0.28[h]	2.57±0.36[h]	3.26±0.11[i]	2.55±0.52[hi]
Σ pigments	Kulon	16.70[j]	15.81[j]	15.42[j]	22.13[k]
	Yakhont	15.52[j]	19.67[k]	20.42[k]	22.18[k]
Chl (a + b) in LHS, % of total Chl	Kulon	57.04	61.72	54.44	58.93
	Yakhont	55.90	63.57	55.88	56.50
Dry matter content in 1 g, %	Kulon	10.24±0.37	9.78±0.27	10.90±0.54	10.18±0.57
	Yakhont	9.95±0.30	9.27±0.31	11.16±0.74	10.42±0.45

Table 2: The effect of seed-pretreatment with 24-epibrassinolide (24-EB) on the dry matter content in 1 g (%), photosynthetic pigments content (mg/g dry weight) and the quantity of chlorophylls in the light harvesting complex (% of total chlorophylls) in leaves of tomato plants under 22 °C and after chilling stress (2 °C for 24 h) Mean values ± SE, Different lowercase letters (a, b, c …) indicate significant differences between treatments (P ≤ 0.05) according to Student's t-test of unpaired samples

Effect of Chilling Stress on Pigment Composition of Leaves

Determined that in chilling-sensitive plants, under the action of chilling stress, the process of photosynthesis is inhibited primarily [1]. Chilling stress often causes severe damage to the structure of chloroplasts [18]. The effect of the pre-sowing treatment of seeds with 24-EB on the pigment composition of tomato leaves under chilling stress (2 °C for 24 h) are presented in Table 2. According to the data, the control plants (without 24-EB) of both cultivars did not differ in the content of pigments at 22 °C. However, pre-sowing treatment of seeds with 24-EB in Yakhont, the content of Chl a and Chl b was 23 and 29 % higher than in Kulon. After chilling stress, the content of chlorophylls in Kulon decreased slightly, and in the variant with 24-EB increased. In Yakhont the content of chlorophylls increased in all variants. At 22 °C, the quantity of chlorophylls in the LHC increased in the 24-EB variant, but decreased under stress (less with 24-EB application). The same changes associated with an increase in the chlorophylls content were observed in pepper plants sprayed with a 24-EB solution before chilling stress [19]. The tomato cultivars did not differ in the content of carotenoids at 22 °C (Table 2). After chilling stress, the content of carotenoids in the leaves of Yakhont (without 24-EB) increased by 26 %. After chilling stress in Kulon (with 24-EB) the content of carotenoids increased by 50%, while in Yakhont it remained at the level of uncooled plants. The use of 24-EB led to more pronounced changes in the pigment composition (total Chl/pigments) in Yakhont in the absence of chilling stress, whereas in Kulon under chilling stress. There is information in the literature that 24-EB application enhances the content of carotenoids in Brassica plant leaves [20]. It is known that 24-EB could prevent loss of photosynthetic pigments, for example, by activation of enzymes that participate in chlorophyll biosynthesis [21]. The data on the pigment composition in tomatoes of Yakhont indicate a reduced sensitivity of their photosynthetic apparatus to chilling stress.

Effect of Chilling Stress on Lipid Peroxidation

Chilling stress could disrupt the cellular homeostasis of the cells and accelerate the production of ROS, which leads to produces toxic products such as MDA is one of the final products of polyunsaturated fatty acids peroxidation [6,8]. It was observed that under 22 °C both tomato cultivars did not differ in the MDA content in the leaves of the control (without 24-EB) plants (Figure 2a). In the variant with 24-EB, the Kulon showed a significant decrease in the lipid peroxidation (by 40%) In this study, we discovered that MDA content significantly enhanced (by about 40%) in Kulon (without 24-EB) by chilling stress (2 °C for 24 h), showing that the plasma membrane was affected and lipid peroxidation increased. At the same time, the plants of Yakhont did not show significant difference in terms of MDA content. After chilling stress in the variants with 24-EB, the MDA content in the leaves of Kulon decreased, whereas in Yakhont did not show a significant difference. Thus, the data on the intensity of oxidative processes indicate a different reaction of tomato cultivars to 24-EB and chilling stress. The plants of Kulon showed greater responsiveness, while the Yakhont reacted with restraint to the application of 24-EB. The results also indicated that 24-EB treatment could improve the cold resistance of Kulon through suppressing the lipid peroxidation and the electrolyte leakage from leaves. It can be assumed that Yakhont is more resistant to chilling stress, while Kulon – as less cold-resistant.

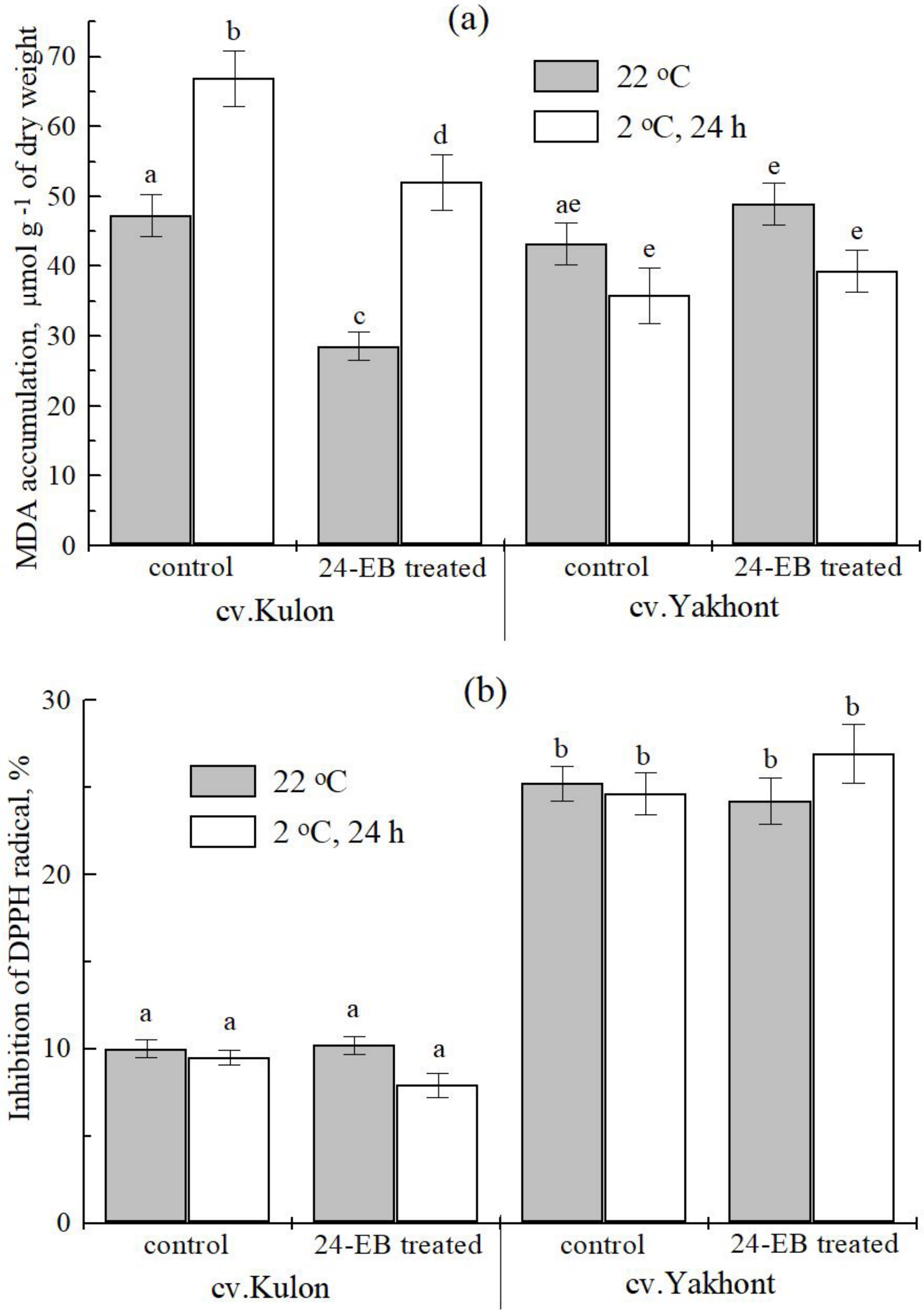

Figure 2: The effect of seed-pretreatment with 24-epibrassinolide (24-EB) on lipid peroxidation (a) and overall antioxidant activity (by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging) (b) in tomato plants at 22 °C and after chilling stress (2 °C for 24 h). Each value represents the mean ± SE. Different lowercase letters (a, b, c …) above bare indicate significant differences between treatments (P ≤ 0.05)

Effect of Chilling Stress on Antioxidant Activity of Leaf Extracts

We assumed that the increased cold resistance of Yakhont is associated with the presence of a more effective antioxidant system in them than in Kulon. We determined the AOA of leaf extracts by a method based on the interaction of a stable chromogen radical DPPH with substances (thiols, phenolic compounds, ascorbic acid, etc.) exhibiting AOA [22]. According to the method, the higher of inhibition of DPPG radicals (%), and the higher the AOA of plant tissue. The AOA of leaf extracts in Yakhont was twice as high as in the Kulon under all experimental conditions (with/without 24-EB treatment, 22 ^{o}C/2 ^{o}C for 24 h) (Figure 2b). We have not established the effect of 24-EB on the level of low molecular weight antioxidants, which indicates the possible effect of this growth regulator on the enzymes of the antioxidant system. There is information in the literature that pepper plants treated with 24-EB had higher activities of antioxidant enzymes and their tissues contained less MDA at chilling stress [19]. 24-EB improved the grapevine's antioxidant defense system during chilling stress [23], and also maintains a balance between ROS and antioxidants [24].

Conclusions

To sum up, the main results and conclusions are as followings:

1 The pretreatment of seeds with 24-EB increased resistance of tomato plants to chilling stress.
2 Tolerance to chilling stress in Yakhont was higher than that of Kulon.
3 The less pronounced reaction of Yakhont to the use of 24-EB and chilling stress is due to the presence of a more effective non-enzymatic antioxidant system and, consequently, their genetically determined by a higher cold resistance than that of Kulon.

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