EVALUATION OF HIGH TEMPERATURE TOLERANCE AND PHYSIOLOGICAL RESPONSES OF DIFFERENT ANOECTOCHILUS GERMPLASM RESOURCES

MEI, Y.[1,2,3#] – QIU, D.[2,3#] – XIAO, S.[1,*] – CHEN, D.[1,4*]

[1]*Horticulture and Landscape College, Hunan Agricultural University, Changsha 410128, China*

[2]*Crops Research Institute, Guangdong Academy of Agricultural Sciences Guangzhou 510640, China*

[3]*Key Laboratory of Crops Genetics and Improvement of Guangdong Province Guangzhou 510640, China*

[4]*Guangdong Key Laboratory of Tea Plant Resources Innovation and Utilization Guangzhou 510640, China*

**Corresponding authors*
e-mail: 23952650030@qq.com (S. Xiao), dongchen1113@sohu.com (D. Chen)

[#]*These authors contributed equally to this work.*

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Abstract. *Anoectochilus* genus is a precious Chinese medicinal plant. High temperature is among the critical factors that threatens plant growth in the *Anoectochilus* genus. The experiment was conducted at Key Laboratory of Crops Genetics and Improvement of Guangdong Province, Guangzhou, China. Two lab experiments were conducted to evaluate the high temperature tolerance of different *Anoectochilus* germplasm resource and the physiological responses of high-temperature-resistant and sensitive *Anoectochilus* to high temperature. Heat injury index, recovery index, soluble protein contents, proline content, malondialdehyde (MDA) content, relative electrical conductivity (REC), superoxide dismutase (SOD) activity, and catalase (CAT) activity were investigated. The results showed that, the recovery index was strongly negatively correlated with the heat injury index. The mean heat injury index at 6 h, 12 h, 24 h, 30 h and 36 h under high temperature were 5.17%, 16.90%, 39.96%, 53.70% and 70.61%, respectively. Five high-temperature-resistant species of *Anoectochilus* were selected. Compared with high-temperature-sensitive species of *Anoectochilus* germplasm, the high-temperature-resistant *Anoectochilus* germplasm had higher SOD activity, proline content and lower REC, CAT activity, MDA, and soluble protein content. REC, SOD activity and soluble protein content were strongly related to heat injury index. In conclusion, REC, SOD activity and soluble protein content are important attributes that could help to screen out the high temperature tolerant varieties of *Anoectochilus*.
Keywords: *Anoectochilus, high temperature stress, heat injury index, physiological indexes, enzymatic activity*

Introduction

Anoectochilus is an important medicinal plant species in China and recognized as ‘golden grass’ and/or ‘the king of medicine’ due to its high medicinal value. It has notable anti-inflammatory effects (Zhang et al., 2007; Du et al., 2008). *Anoectochilus* plants generally have substantial concentrations of polysaccharides, alkaloids, flavonoids, amino acids and organic acids etc. (Zheng et al., 2013; Shao et al., 2014). The extracts of *Anoectochilus* had been reported to cure diabetes, hypertension, tumor, nephritis, fever, snakebite, chest and abdominal pain etc. (Du et al., 2003; Fang et al., 2008). Therefore, *Anoectochilus* was endowed with high edible, medicinal as well as ornamental value.

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The *Anoectochilus* species required cool, humid, low-light environment for its optimum growth. Generally, most of the *Anoectochilus* species were sensitive to high temperature. The optimum temperature for the growth of *Anoectochilus* was ranging from 18 °C to 25 °C, and ≥ 70% relative humidity whereas temperature over 30 °C inhibited the growth of *Anoectochilus*. So, it was important to enhance the heat resistance for the introduction and breeding of new *Anoectochilus* varieties. However, the high-temperature-resistance ability of the different *Anoectochilus* species are still unknown. Moreover, no study has yet been reported the evaluation of the high-temperature-resistance ability of the different *Anoectochilus* species.

How does the *Anoectochilus* respond under high temperature? Like other plants, the *Anoectochilus* plant might activate some physiological and biochemical responses to avoid high temperature damage (He et al., 2004). MDA, proline and soluble protein are important osmoregulator substances in plants under stress conditions. Enhancement in the activities of antioxidants such as superoxide dismutase (SOD) and catalase (CAT) improved plant stress tolerance. For example, Wang et al. (2018) indicated that amino acids and soluble proteins are important for Hard Fescue to resist heat stress. For lettuce seedlings, the malondialdehyde (MDA), proline and soluble sugar contents in leaf were associated with heat stress (Han et al., 2013). The activities of SOD, CAT and peroxidase (POD), as well as the contents of MDA in *Rhododendron lapponicum* leaves were assumed as key physiological indexes in response to heat stress (Li et al., 2018). Rivero et al. (2015) reported that high temperature stress on tomato plants would cause the increase of SOD whilst decrease in CAT activities. Zhang et al. (2017) suggested that *C. nitidissima* plants might have some level of tolerance to high temperature period that might be related to the growing period of the plants. Chen et al. (2016) showed that the leaves of gerbera cultivars had a phenotypic response to heat treatment, as a consequence of increased relative electrical conductivity (REC), decreased soluble protein, and accumulated MDA and proline in leaves. Tian et al. (2011) reported that the marigold cultivars increased SOD, POD and CAT activities, and increased MDA contents and REC in response to high temperature stress. To date, very few studies had been conducted to investigate the physiological responses of *Anoectochilus* under high-temperature stress. Therefore, present study was aimed to establish a method to evaluate high-temperature-resistance of *Anoectochilus* species by evaluating the heat injury index and recovery index of different *Anoectochilus* species, and to investigate the physiological responses of *Anoectochilus* under high-temperature stress.

Materials and methods

Plant materials and growth

Thirty-two *Anoectochilus* genus species were collected from different provinces of China (*Table 1*) by the Southern Medicine Research Group of Crop Research Institute, Guangdong Academy of Agricultural Sciences. The plant samples were grown under greenhouse conditions for 6 months. Uniform seedlings with 8~9 cm height, 3~4 leaves, and 2~3 healthy roots were selected. The seedlings were then transplanted to a 6 cm × 6 cm × 6 cm plastic planting container, 1 plant per container. For high temperature treatment, the container was filled with cultivation substrates (peat: vermiculite = 1:2 (V:V)).

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Table 1. *The resource information of the 32 species of Anoectochilus genus*

Species	Time of collection	Location
NYJ1	2013.08	Fuzhou, Fujian
NYJ2	2013.08	Fuzhou, Fujian
NYJ3	2013.08	Fuzhou, Fujian
NYJ4	2013.08	Fuzhou, Fujian
NYJ5	2010.06	Fuzhou, Fujian
NYJ6	2013.08	Fuzhou, Fujian
NYJ7	2010.06	Fuzhou, Fujian
NYJ8	2013.07	Qujing, Yunnan
NYJ9	2013.07	Qujing, Yunnan
NYJ10	2014.05	Guangzhou, Guangdong
NYJ11	2010.06	Fuzhou, Fujian
NYJ12	2013.08	Fuzhou, Fujian
NYJ13	2012.09	Nankunshan, Guangdong
NYJ14	2012.09	Nankunshan, Guangdong
NYJ15	2012.09	Nankunshan, Guangdong
NYJ16	2014.11	Shenzhen, Guangdong
NYJ17	2013.08	Fuzhou, Fujian
NYJ18	2014.11	Heyuan, Guangdong
NYJ19	2014.11	Heyuan, Guangdong
NYJ20	2015.1	Longyan, Fujian
NYJ21	2015.1	Longyan, Fujian
NYJ22	2014.12	Jinxiu, Guangxi
NYJ23	2014.12	Jinxiu, Guangxi
NYJ24	2015.1	Sanming, Fujian
NYJ25	2013.08	Fuzhou, Fujian
NYJ26	2015.1	Sanming, Fujian
NYJ27	2015.1	Longyan, Fujian
NYJ28	2015.12	Heyuan, Guangdong
NYJ29	2015.12	Qingyuan, Guangdong
NYJ30	2016.03	Mangshi, Yunnan
NYJ31	2015.1	Beiliu, Guangxi
NYJ32	2015.1	Meizhou, Guangdong

Experiment 1: High temperature treatment for thirty-two Anoectochilus genus species

High temperature treatment stress was imposed at Key Laboratory of Crops Genetics and Improvement of Guangdong Province, Crop Research Institute of Guangdong Academy of Agricultural Sciences. The high temperature treatment processes were as follows: before high temperature treatment, all plants were grown in an artificial climate incubator with a day/night temperature of 25 °C /18 °C, light intensity of 2000 Lx 12 h day/night cycle, and relative humidity of 80% for 15 days. The plants were watered

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once per day at 20 ml per plant. Then, the plants were transferred to incubator with day/night temperature of 43 °C /38 °C, illumination intensity of 2000 Lx 12 h day/night cycle, and relative humidity of 80%. The heat injury index of different species of *Anoectochilus* were determined at 6 h, 12 h, 24 h, 30 h, and 36 h as described by Li et al. (2009). After that, all the plants were subjected to incubation with a day/night temperature of 25 °C /18 °C, light intensity of 2000 Lx 12 h day/night cycle, and relative humidity of 80% for 15 days and the recovery index was investigated according to Jia and Chen (2005). The heat injured plants were separated into five levels: level 0, no injured leaves, green; level 1, drooping leaves, leaves' color were normal; level 2, the tip of the leaves wilted; level 3, 50% of the leaves wilted; level 4, almost all the leaves wilted; level 5, the whole plant died. The calculation of heat injury index was according to the following formula: heat injury index = ∑(number of plant at different injury level × injury level) injury level / (the highest injury level × total number of plants) × 100%. For calculation of recovery index, the plants were separated into different levels: level 0, the whole plant died; level 1, stems kept erecting, dry leaves, plants were unable to recover; level 3, the whole plant recovered and grew well. The recovery index was calculated as the following formula: recovery index = ∑(number of plant at different recovery level × recovery level) injury level / (the highest recovery level × total number of plants) × 100%.

Experiment 2: Physiological responses of high-temperature-resistant and sensitive Anoectochilus

The fresh plant sample (5.0 g) of all the plant species was collected at 6 h, 12 h, 24 h, 30 h, and 36 h under high temperature treatment, soaked in liquid nitrogen for 1 min, and then immediately stored at -80 °C for the determination of physiological indexes. The physiological indexes, i.e. soluble protein contents, proline content, malondialdehyde (MDA) content, relative electrical conductivity (REC), and the activities of superoxide dismutase (SOD) and catalase (CAT) were determined to test the high-temperature-resistance and sensitivity of *Anoectochilus*.

Measurement of soluble protein contents

Soluble protein contents were estimated according to the methods devised by Bradford (1976) using G-250, the absorbance was read at 595 nm and the concentration was expressed as $mg \cdot g^{-1}$.

Determination of proline content

Proline contents were determined according to the method of Bates et al. (1973) by evaluating the absorbance of the red chromophore in the toluene fraction at 520 nm and the amount of proline was estimated by comparing with a standard curve and expressed as $\mu g \cdot g^{-1}$.

Determination of malondialdehyde (MDA) content

The malondialdehyde (MDA) content was measured by the method of Chen and Wang (2006). MDA reacted with thiobarbituric acid (TBA), the absorbance of the reaction solutions were recorded at 532 nm, 600 nm, and 450 nm. The final result of MDA was expressed as nmol·g.

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Determination of relative electrical conductivity (REC)

The plant leaves were thoroughly washed with deionized water. The leaf sample was then put into a clean beaker with 30 ml of deionized water and kept under vacuum for 15 min. The electrical conductivity was measured and marked as EC1. The leaves were then heated in boiling water for 20 min and then cooled at room temperature. The electrical conductivity was investigated again and marked as EC2. The formula for the calculation of the relative electrical conductivity (REC) calculation was: REC = (EC1/EC2) × 100%.

Determination of superoxide dismutase (SOD) activity

The superoxide dismutase activity was measured by using Nitro blue tetrazolium (NBT) method, after the reaction, the color change was measured at 560 nm, and one unit of SOD activity was equal to the volume of extract needed to cause 50% inhibition of the color reaction. The enzyme activity was expressed as $U \cdot g^{-1}$ (Wang et al., 2009).

Determination of catalase (CAT) activity

Catalase activity was determined by the method of Wang et al. (2009), 50 μl of enzyme extract was added to the reaction solution system containing 1 ml 0.3% H_2O_2 and 1.95 ml H_2O, the absorbance change at 240 nm was recorded for calculating CAT activity. The enzyme activity was expressed as $U \cdot g^{-1}$.

Statistical analysis

The data were analyzed using statistical software SPSS V 18.1; the differences amongst means were separated by using least significant difference (LSD) test at 5% probability level. Microsoft Excel was used for graphical representation.

Results

Experiment 1

Heat injury index and recovery index

The heat injury index investigated at 6 h, 12 h, 24 h, 30 h and 36 h, under high temperature treatment and the recovery index at 15 d after high temperature treatment was shown in *Table 2*. The heat injury index in different species of *Anoectochilus* was different, a wide range of heat injury index was detected at 6 h (0. 00%- 15.33%), 12 h (0. 00%- 58.00%), 24 h (2.00%- 74.67%), 30 h (2.00- 78.00%) and 36 h (37.33-94.00%) under high temperature. The mean heat injury index at 6 h, 12 h, 24 h, 30 h and 36 h under high temperature were 5.17%, 16.90%, 39.96%, 53.70% and 70.61%, respectively. At 30 h of high temperature treatment, over 75% of the *Anoectochilus* species had a heat injury index higher than 50%. At 36 h of high temperature treatment, over 80% of the *Anoectochilus* species had a heat injury index higher than 60.00%. The recovery index at 15 d after recovery was ranging from 0.00% to 74.44%, with a mean of 23.85%. Over 80% of the *Anoectochilus* species had a recovery index lower than 40.00%. Overall, the *Anoectochilus* species, like NYJ2, NYJ6, NYJ14, NYJ21 and NYJ24 were high-temperature-resistant species of *Anoectochilus*, and others were high-temperature-sensitive species of *Anoectochilus*.

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Table 2. *The heat injury index and recovery index of 32 species of Anoectochilus genus under heat stress*

Species	Heat injury index					Recovery index
	6 h	**12 h**	**24 h**	**30 h**	**36 h**	**15 d**
NYJ1	7.33	11.33	45.33	59.33	74.00	22.22
NYJ2	0.00	0.67	2.00	14.00	40.67	74.44
NYJ3	8.67	18.07	38.00	58.67	67.33	38.89
NYJ4	6.67	24.67	49.33	62.00	76.67	22.22
NYJ5	7.33	20.00	50.00	68.00	79.33	0.00
NYJ6	0.00	0.00	2.00	12.67	37.33	67.78
NYJ7	8.67	22.67	51.30	63.33	75.33	5.56
NYJ8	4.00	18.00	42.00	53.33	67.33	31.11
NYJ9	6.00	22.00	47.33	60.67	77.33	17.78
NYJ10	4.67	14.00	40.67	52.67	64.00	28.89
NYJ11	6.67	14.67	58.67	62.00	80.67	5.56
NYJ12	6.00	24.67	46.67	61.33	78.00	21.11
NYJ13	2.67	10.00	36.67	39.33	66.67	34.44
NYJ14	0.67	0.67	4.00	15.33	39.33	60.00
NYJ15	3.33	16.00	38.00	41.00	64.67	32.22
NYJ16	4.67	24.00	39.33	62.00	73.33	12.22
NYJ17	3.33	12.67	36.67	54.67	70.67	30.00
NYJ18	15.33	54.00	77.33	74.00	88.66	0.00
NYJ19	12.67	58.00	74.67	71.33	87.33	0.00
NYJ20	6.00	10.67	46.00	68.00	91.00	4.44
NYJ21	0.67	2.00	2.67	15.33	44.67	51.11
NYJ22	10.60	16.67	46.00	67.33	94.00	3.33
NYJ23	1.33	16.00	47.33	63.33	72.00	31.11
NYJ24	0.67	4.00	6.00	15.33	42.00	44.44
NYJ25	8.00	21.33	48.00	68.00	84.67	0.00
NYJ26	2.00	14.67	42.67	66.00	74.00	12.22
NYJ27	9.33	18.67	45.33	68.00	83.33	2.22
NYJ28	2.00	8.00	34.00	59.33	70.67	33.33
NYJ29	1.33	14.00	42.00	59.33	68.00	31.11
NYJ30	2.67	13.33	48.66	56.00	69.33	31.11
NYJ31	9.33	22.67	50.67	64.00	80.67	0.00
NYJ32	2.67	12.67	39.33	62.67	76.67	14.44
Mean	5.17	16.90	39.96	53.70	70.61	23.85

Experiment 2

From the 32 species, one high-temperature-resistant and one high-temperature-sensitive *Anoectochilus* was selected to investigate the physiological response of the different *Anoectochilus*.

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Relative electrical conductivity (REC)

Before high temperature treatment, the two species had similar levels of REC. With high temperature treatment, NYJ2 had lower REC than NYJ18. At 6 h, 12 h, 24 h, 30 h and 36 h under high temperature treatment, the REC was significantly higher than CK for NYJ2. For NYJ18, at 6 h under high temperature treatment, the REC level was slightly higher than CK, while at 12 h, 24 h, 30 h and 36 h under high temperature treatment, the REC increased rapidly and significantly and it was higher than CK (*Fig. 1*).

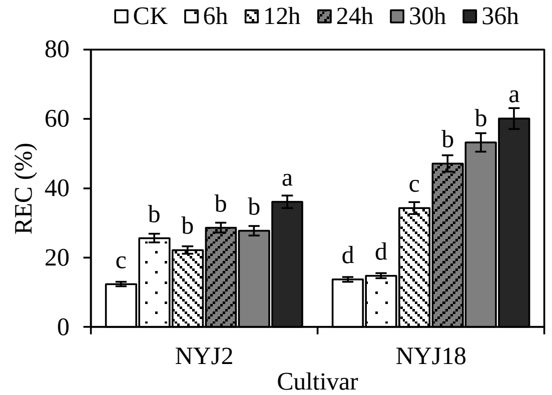

***Figure 1.** Effect of high temperature stress on the relative electrical conductivity (REC) in high-temperature-resistant Anoectochilus germplasm (NYJ2) and high-temperature-sensitive Anoectochilus germplasm (NYJ18). Vertical bars with different lower case letters above are significantly different at P = 0.05 by LSD tests. Capped bars represent SD*

Superoxide dismutase (SOD) activity

For NYJ2, the SOD activity at 6 h, 12 h, 24 h and 30 h under high temperature treatment was significantly higher than CK. At 36 h under high temperature treatment, the SOD activity was similar to that in CK. For NYJ18, compared with CK, the SOD activity at 6 h, 12 h, 24 h and 36 h under high temperature treatment was significantly decreased. The SOD activity at 36 h under high temperature treatment was slightly decreased. The SOD activity in NYJ2 was higher than in NYJ18 (*Fig. 2*).

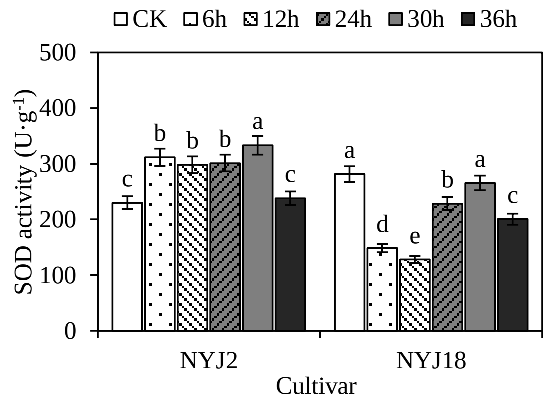

***Figure 2.** Effect of high temperature stress on SOD activity in high-temperature-resistant Anoectochilus germplasm (NYJ2) and high-temperature-sensitive Anoectochilus germplasm (NYJ18). Vertical bars with different lower case letters above are significantly different at P = 0.05 by LSD tests. Capped bars represent SD*

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Catalase (CAT) activity

The CAT activity in NYJ2 was lower than in NYJ18. The CAT activity at 6 h, 12 h under high temperature treatment was slightly decreased for NYJ2, whilst the CAT activity at 24 h, 30 h and 36 h under high temperature treatment was dramatically decreased. For NYJ18, short time high temperature treatment (6 h) did not affect CAT activity, but at 12 h, 24 h, 30 h, and 36 h high temperature treatment, the CAT activity was decreased linearly (*Fig. 3*).

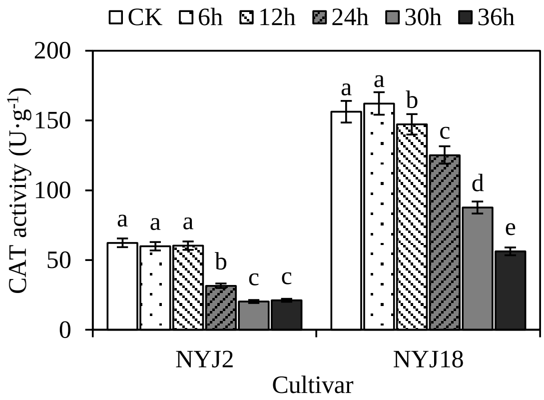

***Figure 3.** Effect of high temperature stress on CAT activity in high-temperature-resistant Anoectochilus germplasm (NYJ2) and high-temperature-sensitive Anoectochilus germplasm (NYJ18). Vertical bars with different lower case letters above are significantly different at P = 0.05 by LSD tests. Capped bars represent SD*

Malondialdehyde (MDA) content

The MDA content in NYJ2 was lower than in NYJ18. As temperature increased, the MDA content in the plant was increased. For NYJ2, in the 6 h high temperature treatment the MDA content was slightly increased, in the 30 h high temperature treatment the MDA content reached a peak, then decreased in the 36 h high temperature treatment. For NYJ18, the MDA content increased gradually when high temperature treatment time increased (*Fig. 4*).

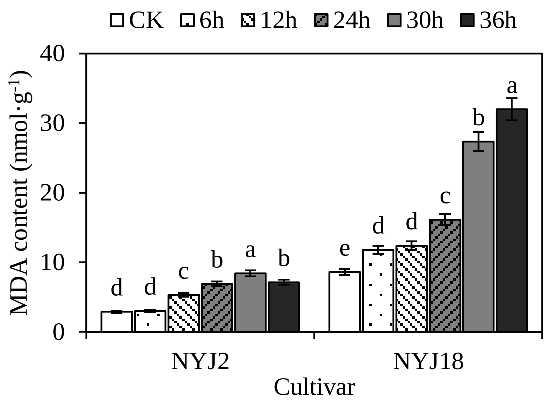

***Figure 4.** Effect of high temperature stress on malondialdehyde (MDA) content in high-temperature-resistant Anoectochilus germplasm (NYJ2) and high-temperature-sensitive Anoectochilus germplasm (NYJ18). Vertical bars with different lower case letters above are significantly different at P = 0.05 by LSD tests. Capped bars represent SD*

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Proline content

With increase in temperature, the proline content in NYJ2 increased gradually, and was significantly higher than CK. For NYJ18, the proline content was significantly decreased in the 6 h high temperature treatment only. At 12 h, 24 h 30 h, and 36 h under high temperature treatment, the proline content was substantially improved (*Fig. 5*).

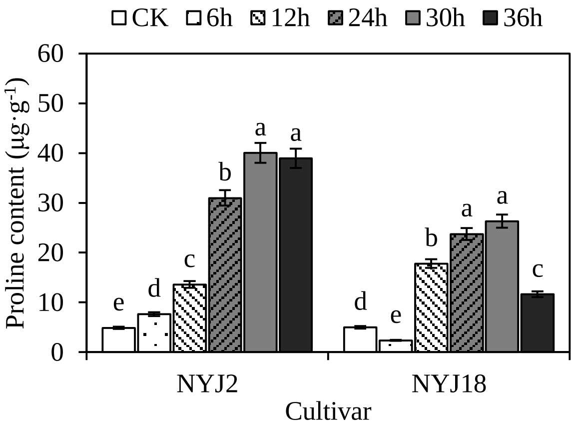

Figure 5. *Effect of high temperature stress on proline content in high-temperature-resistant Anoectochilus germplasm (NYJ2) and high-temperature-sensitive Anoectochilus germplasm (NYJ18). Vertical bars with different lower case letters above are significantly different at P = 0.05 by LSD tests. Capped bars represent SD*

Soluble protein content

Exposure of high temperature stress for short duration (6 h, 12 h and 24 h) did not significantly affect soluble protein content in NYJ2. At 30 h and 36 h high temperature treatment, the soluble protein content was significantly increased in NYJ2 whereas, for NYJ18, at 6 h high temperature treatment, the soluble protein content was decreased significantly. With increased duration of high temperature, the soluble protein contents were increased (*Fig. 6*).

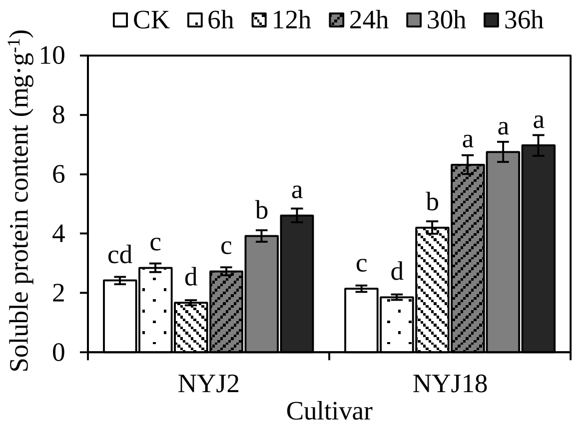

Figure 6. *Effect of high temperature stress on soluble protein content in high-temperature-resistant Anoectochilus germplasm (NYJ2) and high-temperature-sensitive Anoectochilus germplasm (NYJ18). Vertical bars with different lower case letters above are significantly different at P = 0.05 by LSD tests. Capped bars represent SD*

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Correlation analysis

The recovery index showed a significant negative correlation relationship with heat injury index at 6 h (-0.7675**), 12 h (-0.6687**), 24 h (-0.8566**), 30 h (-0.8894**), and 36 h (-0.9291**), respectively (*Table 3*). The heat injury index was significantly associated with REC (0.9186**), SOD activity (0.8699**), and soluble protein content (0.9442**) for both high-temperature-resistant and sensitive *Anoectochilus* germplasm (*Table 4*). For high-temperature-resistant *Anoectochilus* germplasm (NYJ2), a significant positive correlation relationship between heat injury index and soluble protein content (0.8741*) was investigated (*Table 5*). For high-temperature-sensitive *Anoectochilus* germplasm (NYJ18), the heat injury index was significantly associated with REC (**0.9783****), MDA content (-0.8241*), and soluble protein content (**0.9695****) (*Table 6*).

Table 3. *Correlation analysis between heat injury index and recovery index*

Investigated index	Recovery index at 15d	Heat injury index 6 h	Heat injury index 12 h	Heat injury index 24 h	Heat injury index 30 h	Heat injury index 36 h
Recovery index at 15 d	1.0000	-0.7675**	-0.6687**	-0.8566**	-0.8894**	-0.9291**
Heat injury index 6 h	-0.7675**	1.0000	0.8167**	0.7813**	0.6926**	0.7623**
Heat injury index 12 h	-0.6687**	0.8167**	1.0000	0.8292**	0.6653**	0.6636**
Heat injury index 24 h	-0.8566**	0.7813**	0.8292**	1.0000	0.9171**	0.9021**
Heat injury index 30 h	-0.8894**	0.6926**	0.6653**	0.9171**	1.0000	0.9519**
Heat injury index 36 h	-0.9291**	0.7623**	0.6636**	0.9021**	0.9519**	1.0000

Significant correlations at **$p < 0.01$

Table 4. *Correlation analysis between the investigated indexes for both high-temperature-resistant and sensitive Anoectochilus germplasm*

Investigated index	Heat injury index	REC (%)	SOD activity ($U \cdot g^{-1}$)	CAT activity ($U \cdot g^{-1}$)	MDA content (nmol/g)	Proline content ($\mu g \cdot g^{-1}$)	Soluble protein content ($mg \cdot g^{-1}$)
Heat injury index	1.0000	0.9186**	0.8699**	-0.4571	0.1295	0.2410	0.9442**
REC (%)	0.9186**	1.0000	0.8417**	-0.1427	-0.1792	0.3998	0.9554**
SOD activity ($U \cdot g^{-1}$)	0.8699**	0.8417**	1.0000	-0.3337	0.1518	0.0502	0.8279**
CAT activity ($U \cdot g^{-1}$)	-0.4571	-0.1427	-0.3337	1.0000	-0.6031*	0.3406	-0.1872
MDA content (nmol/g)	0.1295	-0.1792	0.1518	-0.6031*	1.0000	-0.5853*	-0.1062
Proline content ($\mu g \cdot g^{-1}$)	0.2410	0.3998	0.0502	0.3406	-0.5853*	1.0000	0.4018
Soluble protein content ($mg \cdot g^{-1}$)	0.9442**	0.9554**	0.8279**	-0.1872	-0.1062	0.4018	1.0000

Significant correlations at *$p < 0.05$ and **$p < 0.01$

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Table 5. *Correlation analysis between the investigated indexes for high-temperature-resistant Anoectochilus germplasm*

Investigated index	**Heat injury index**	**REC (%)**	**SOD activity ($U \cdot g^{-1}$)**	**CAT activity ($U \cdot g^{-1}$)**	**MDA content ($nmol \cdot g^{-1}$)**	**Proline content ($\mu g \cdot g^{-1}$)**	**Soluble protein content ($mg \cdot g^{-1}$)**
Heat injury index	1.0000	0.7381	0.5615	-0.3608	-0.7247	0.7130	0.8741*
REC (%)	0.7381	1.0000	0.6988	0.2077	-0.7803	0.7974	0.7244
SOD activity ($U \cdot g^{-1}$)	0.5615	0.6988	1.0000	0.3369	-0.9155*	0.9620**	0.5820
CAT activity ($U \cdot g^{-1}$)	-0.3608	0.2077	0.3369	1.0000	-0.1482	0.2003	-0.1143
MDA content ($nmol \cdot g^{-1}$)	-0.7247	-0.7803	-0.9155*	-0.1482	1.0000	-0.9876**	-0.8287*
Proline content ($\mu g \cdot g^{-1}$)	0.7130	0.7974	0.9620**	0.2003	-0.9876**	1.0000	0.7665
Soluble protein content ($mg \cdot g^{-1}$)	**0.8741***	0.7244	0.5820	-0.1143	-0.8287*	0.7665	1.0000

Significant correlations at *p < 0.05 and **p < 0.01

Table 6. *Correlation analysis between the investigated indexes for high-temperature-sensitive Anoectochilus germplasm*

Investigated index	**Heat injury index**	**REC (%)**	**SOD activity ($U \cdot g^{-1}$)**	**CAT activity ($U \cdot g^{-1}$)**	**MDA content ($nmol \cdot g^{-1}$)**	**Proline content ($\mu g \cdot g^{-1}$)**	**Soluble protein content ($mg \cdot g^{-1}$)**
Heat injury index	1.0000	0.9783**	0.8082	-0.0252	-0.8241*	0.7524	0.9695**
REC (%)	**0.9783****	1.0000	0.8901*	0.1310	-0.9126*	0.7246	0.9904**
SOD activity ($U \cdot g^{-1}$)	0.8082	0.8901*	1.0000	0.1805	-0.9831**	0.4295	0.8452*
CAT activity ($U \cdot g^{-1}$)	-0.0252	0.1310	0.1805	1.0000	-0.2541	0.2149	0.2168
MDA content ($nmol \cdot g^{-1}$)	**-0.8241***	-0.9126*	-0.9831**	-0.2541	1.0000	-0.4417	-0.8757*
Proline content ($\mu g \cdot g^{-1}$)	0.7524	0.7246	0.4295	0.2149	-0.4417	1.0000	0.7895
Soluble protein content ($mg \cdot g^{-1}$)	**0.9695****	0.9904**	0.8452*	0.2168	-0.8757*	0.7895	1.0000

Significant correlations at *p < 0.05 and **p < 0.01

Discussion

In this study, we applied the high temperature treatment method to evaluate the high-temperature-resistance of *Anoectochilus* species. In this method, the recovery index was strongly negatively correlated with heat injury indices for *Anoectochilus* species (*Table 3*). The mean heat injury index at 6 h, 12 h, 24 h, 30 h and 36 h under high temperature were 5.17%, 16.90, 39.96%, 53.70% and 70.61%, respectively. Over 80% of the *Anoectochilus* species were high-temperature-sensitive, five high-temperature-resistant species of *Anoectochilus* (NYJ2, NYJ6, NYJ14, NYJ21 and NYJ24) were identified (*Table 2*).

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The REC and MDA content of the two species of *Anoectochilus* genus were apparently increased with the increase of high temperature stress. Han et al. (2013) indicated that high temperature could lead to increased membrane permeability and membrane lipid peroxidation. The REC increased as a consequence of increasing membrane permeability which led to extracellular electrolyte leakage. In our study, REC and MDA content of the high-temperature-sensitive NYJ18 increased more than that of high-temperature-resistant NYJ2.

In addition, when both high-temperature-resistant *Anoectochilus* germplasm resource (NYJ2) and high-temperature-sensitive *Anoectochilus* germplasm resource (NYJ18) were pooled together, the heat injury index significantly associated to REC (0.9186**). For high-temperature-sensitive *Anoectochilus* germplasm resource (NYJ18), the heat injury index significantly associated to REC (0.9783**) and MDA content (-0.8241*). It could be seen that the heat injury index of different breedings of *Anoectochilus* were significantly positively correlated with REC. The high-temperature-sensitive species had strong negative relation to MDA content (Peng et al., 2012). Therefore, by comparing the increase range of REC and MDA content of two species to identify their high-temperature-resistant ability, which was consistent with the conclusions of most research on other plants (Lin et al., 2014; Li et al., 2015).

For anti-enzyme activity, SOD could reduce superoxide radicals into hydrogen peroxide, while CAT could trigger the conversion of hydrogen peroxide into water and oxygen (Xu et al., 2006). In this study, the results showed that CAT activity in both species of *Anoectochilus* decreased under heat stress, and in high-temperature-sensitive NYJ18 it decreased more rapidly than in high temperature tolerant NYJ2. The SOD activity of the high-temperature-sensitive NYJ18 decreased rapidly at the beginning of stress treatment; while the SOD activity for high temperature tolerant NYJ2 increased. Moreover, when both high-temperature-resistant *Anoectochilus* germplasm resource (NYJ2) and high-temperature-sensitive *Anoectochilus* germplasm resource (NYJ18) were pooled together, the heat injury index significantly associated with SOD activity (0.8699**). SOD was resistant to membrane lipid peroxidation and maintenance of membrane structure integrity, and these were great significance (Lin et al., 2014). The changes of SOD and CAT activity with high temperature treatment time in this study were consistent with other researches (Jiang et al., 2008; Peng et al., 2012; Can et al., 2016).

Soluble protein and proline in leaves act as osmotic adjustment substances, and are beneficial to the maintenance of cell structure and function under stress. Previous study suggested that soluble protein and proline were active oxygen scavengers, which had a protective effect on plant cells (Can et al., 2016). With time and stress level increased, within a certain temperature range, the accumulation of free proline was positively correlated with the stress intensity (Liu et al., 2001; Sun et al., 2006). In this study, the proline content of the high-temperature-resistant NYJ2 was significantly higher than that of high-temperature-sensitive NYJ18 in the late stress stage and the proline content in the high-temperature-sensitive NYJ18 significantly decreased. In addition, the soluble protein content of NYJ2 and NYJ18 decreased in a certain range at the beginning of stress treatment (before 12 h), and then increased with the prolongation of stress time. The soluble protein content of high-temperature-resistant NYJ2 was significantly lower than that of high-temperature-sensitive NYJ18. Moreover, when we pooled both high-temperature-resistant *Anoectochilus* germplasm resource (NYJ2) and high-temperature-sensitive *Anoectochilus* germplasm resource (NYJ18) together, the

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heat injury index significantly associated with soluble protein content (0.9442**). For high-temperature-resistant *Anoectochilus* germplasm resource (NYJ2), the heat injury index significantly associated with soluble protein content (0.8741*). For high-temperature-sensitive *Anoectochilus* germplasm resource (NYJ18), the heat injury index significantly associated with soluble protein content (0.9695**). Thus, soluble protein content might be important for identifying high-temperature-resistance of *Anoectochilus* species.

Conclusion

In conclusion, the recovery index was strongly negatively correlated with the heat injury index. The mean heat injury index at 6 h, 12 h, 24 h, 30 h and 36 h under high temperature were 5.17%, 16.90, 39.96%, 53.70% and 70.61%, respectively. Over 80% of the *Anoectochilus* species were high-temperature-sensitive, five high-temperature-resistant species of *Anoectochilus* (NYJ2, NYJ6, NYJ14, NYJ21 and NYJ24) were identified. Compared with high-temperature-sensitive species of *Anoectochilus* germplasm, the high-temperature-resistant *Anoectochilus* germplasm had higher SOD activity, proline content and lower REC, CAT activity, MDA, and soluble protein content. The heat injury index was significantly associated with REC, SOD activity, and soluble protein content for both high-temperature-resistant and sensitive *Anoectochilus* germplasm. For high-temperature-resistant *Anoectochilus* germplasm, heat injury index was significantly related to soluble protein content. For high-temperature-sensitive *Anoectochilus* germplasm, the heat injury index was significantly associated with REC, MDA content and soluble protein content. This study is important to understand the heat-resistant mechanism and the breeding of new varieties of *Anoectochilus*. Further studies on molecular basis regarding heat-resistance of *Anoectochilus* is needed.

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