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Cold-Resistant Breeding of *Oenothera speciosa* Using Silico Ion Implantation

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Abstract

***Oenothera speciosa*, belonging to thermophilous plant, cannot overwinter in Beijing. To enhance the overwintering rate of *Oenothera speciosa*, the seeds were treated through silico ion implantation (SII), with five various fluence ranges (1×10^9 - 1×10^{11} ions/cm^2) of 40 MeV and four various fluence ranges (1×10^{10} - 5×10^{11} ions/cm^2) of 35 MeV, respectively. M_1 generations of various SII-treated *Oenothera speciosa* lines can overwinter, and the highest overwinter rate (41.3%) was observed in *Oenothera speciosa* lines treated with 35 MeV and fluence 5×10^{10} ions/cm^2. M_2 and M_3 generations of all treated lines were able to overwinter smoothly. The results indicated that SII treatment can enhance the cold-resistance of *Oenothera speciosa* heritably. Furthermore, physiological indexes including relative electrical conductivity, MDA contents and proline contents of SII-treated *Oenothera speciosa* pot seedlings were detected after low temperature stress. The results revealed that relative electrical conductivities and MDA contents of M_1, M_2 and M_3 generations of SII-treated *Oenothera speciosa* plants were lower than that of control, whereas the proline contents were higher than control in the −5˚C cold stress. Taken together, the cold resistance of SII-treated *Oenothera speciosa* plants was improved, which made it possible to be used as a perennial flower in landscaping in Beijing.**

Keywords

Silico Ion Implantation, *Oenothera speciosa*, Cold Resistant Breeding

1. Introduction

Oenothera speciosa is a species of evening primrose, belonging to the family of *Onagraceae* and genus *oenothera*. It is herbaceous perennial wildflower native to 28 of the lower 48 US states (**Figure 1**). The plants can

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live in a range of habitats with loose and fasting-draining soil and full sun areas, such as rocky prairies, open woodlands, slopes, roadsides, meadows and disturbed areas.

Oenothera speciosa, was introduced in Beijing from Shangfang Garden Limited Company in Shanghai in 2004. However, it cannot overwinter in open field in Beijing, which encouraged us to carry out breeding studies in order to improve its cold resistance. In this study, through silico ion implantation method, many characters were improved significantly, such as ground coverage, florescence, cold hardiness and drought tolerance, resistance to cutting, diseases and pests. By our breeding improving, *Oenothera speciosa* has become an environment-friendly variety, which can be applied as a perennial flower in Beijing (Atkinson et al., 2013).

Ion implantation is one of the methods of mutation breeding. That is using the ionization of atoms (or molecules) accelerated to a certain energy, and then injected them into the plant samples. Ion implantation is of energy deposition effect, quality deposition effect and charge exchange effect (Liu et al., 2010). Recently, ion implantation has been explored as a new method to produce new genetic modifications and physiology changes. In this study, according to the breeding objective and physiological characteristics of this plant, the silicon was selected to implant into the experiment sample, and obtained the cold-resistance *Oenothera speciosa* which can overwinter in Beijing (**Figure 2**).

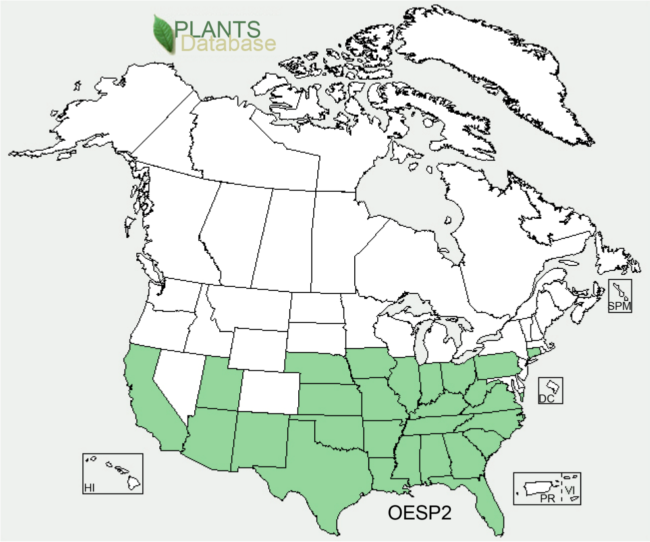

Figure 1. Geographical distribution of *Oenothera speciosa* in USA. (http://plants.usda.gov/core/profile?symbol=OESP2).

Figure 2. Overwintered *Oenothera speciosa* in Beijing.

2. Materials and Methods

2.1. Silico Ion Implantation (SII)

SII was applied on cold-resistant breeding for *Oenothera speciosa* with seeds (Luo et al., 2011; Mahadtanapuk et al., 2013; Wang et al., 2007), at energy of 35 MeV and 40 MeV to a beam fluence range of 1×10^9 - 5×10^{11} ions/cm^2. There are 50 seeds in each group and 2 repeats, total 100 seeds, 10 sample conducted including 1 control (CK: un-treated) and 9 treatments. The detail information for the SII treatment was presented in **Table 1**.

2.2. Seedling Raising and Overwintering Investigation

Trails were performed from 2009 to 2014 (Ma et al., 2012). SII treated seeds of each generation (M_1, M_2 and M_3) and CK were sown in plug trays in green houses in winter. Well-developed seedlings were transferred into pots and cultured as container seedlings, which would be planted in field in next spring, maintained routinely. The green seedlings with 3 - 5 cm in height were calculated in mid-late Apr in the third spring. The overwintering rates were obtained by dividing numbers of planted seedlings by the numbers of green seedlings, then, multiplied by 100.

2.3. Regional Trials of SII-Treated *Oenothera speciosa*

Regional trials were carried out from 2012 to 2014. M_2 and M_3 generation seeds, collected from overwintered green seedlings in 2011 and 2012 respectively, were used to culture container seedlings for regional trials. The testing fields were distributed in Beijing and its suburban districts. The investigations of green seedlings were conducted from mid-late Apr to early May. The overwintering rates were calculated by dividing the numbers of planted seedlings by the numbers of green seedlings in each square meter, then, multiplied by 100. **Table 2** presented the locations, sizes and specific environment of each test field, as well as the corresponding investigating times and overwintering rates.

2.4. The Relationship between the Temperature and Overwintering Rate

The temperature data was collected from automatic weather stations in Beijing Institute of Landscape Architecture. Temperature data were recorded in every hour in Jan, Feb, Mar, Apr, Jun, Nov and Dec from 2012 to 2014. Based on the temperature data, the monthly average temperature and monthly negative accumulated temperature in each month were calculated and the overwintering rates were comparatively analyzed.

2.5. Detections of Physiological Indexes of the SII-Treated *Oenothera speciosa*

Relative electrical conductivity of M_1, M_2, M_3 generations and CK of *Oenothera speciosa* were detected by

Table 1. The average overwintering rate of each generation of SII-treated *Oenother speciosa* in testing years (%).

Sample	Energy (MeV)	Fluence (ions/cm^2)	M_1				M_2			M_3
			2011	2012	2013	2014	2012	2013	2014	2014
CK	0	0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	40	1×10^9	12.9	61.1	8.6	73.1	43.1	45.9	79.2	100.0
2	40	5×10^9	4.9	70.0	26.1	64.7	33.2	91.7	100.0	100.0
3	40	1×10^{10}	11.4	65.0	12.5	73.1	53.9	42.9	100.0	100.0
4	40	5×10^{10}	11.9	55.6	11.6	71.9	57.7	32.0	100.0	100.0
5	40	1×10^{11}	15.3	100.0	26.2	100.0	50.0	40.4	100.0	100.0
6	35	1×10^{10}	25.0	100.0	44.5	82.2	61.5	25.0	100.0	100.0
7	35	5×10^{10}	41.3	100.0	42.9	77.3	61.6	25.0	100.0	100.0
8	35	1×10^{11}	19.6	100.0	28.2	100.0	50.0	84.1	100.0	100.0
9	35	5×10^{11}	39.2	100.0	27.2	100.0	56.9	85.7	100.0	100.0
Average			20.1	93.5	25.3	82.5	52.0	52.5	97.7	100.0

Table 2. Regional tests of cold-resistant M_2 and M_3 generations of *Oenothera speciosa*.

Testing place	Area (m^2)	Environment	Overwintering rate (%)	
			2013	2014
Beihai Pack	500	East slope under the willow	88.0	100.0
Yu Yuantan Park	120	South side of the short wall preventing stepping in winter	93.8	100.0
Bei Xiaohe Garden	1500	Slop, forest land, semi-shading	76.6	100.0
Ditan Park	90	Semi-shading under forest	79.7	100.0
Plant base in Changping	1500	Full sun	84.4	100.0
Jingshan Park	130	Under small trees, semi-shading		100.0
The Heaven Temple	50	Beside over-green tree, semi-shading		100.0
Beijing Zoo	890	Shady slope under large tree, shading and semi-shading		100.0
Beijing Botanical Garden	50	Under small tree, semi-shading		100.0
The Summer Palace	50	Lakeside, full sun		100.0
Liuyin Park	50	Lakeside, semi-shading		100.0

conductivity instrument (Chen et al., 2010; Ye et al., 2012). Malondialdehyde (MDA) contents were detected using thiobarbituric acid (TBS) colorimetric method (Zheng, 2006), and the proline contents were detected using ninhydrin colorimetry method (Zheng, 2006).

The overwintered M_1, M_2 and M_3 *Oenothera speciosa* and un-treated *Oenothera speciosa* plants were sampled for further analysis. Overwintered seedlings were trimmed, transferred to pots and cultured in green house. Since the stems growing to 10 cm in height, the container seedlings were transferred outside. When crown diameters reached to 25 - 30 cm in width, the container seedlings were performed low temperature stress using temperature controlling chambers (RXZ-380C) and ultra-low temperature freezer (Meiling, −86˚C). The treating program was as follow: seven-day treatment for each temperature gradients continuously (0˚C, 10˚C, 5˚C, 0˚C and −5˚C), followed with 90 h for each of ultra-low temperature gradients (−10˚C, −15˚C and −20˚C) (Kalberer et al., 2007; Kim & Anderson, 2006). The leaves of four individual *Oenothera speciosa* plants were harvested separately at each temperature gradient for relative electric conductivity, MDA contents and Porline contents detections. Each group has 12 pots, 3 repeats. Comparison analyses between samples were tested by One-Way ANOVA method.

3. Results and Discussion

3.1. Investigation the Overwintering Rate of SII-Treated *Oenothera speciosa*

The investigation of the overwintering rate was performed between mid-late Apr to early May from 2011 to 2014 (Ma et al., 2012). The survey results were listed in **Table 1**. As **Table 1** shown, each SII-treatment of M_1 *Oenothera speciosa* could obtain the overwintered plants, and their progenies (M_2 and M_3) were overwintered too. But there were no relevance between overwintering rates and the energies and their corresponding fluences which were used in treatments. The highest overwinter rate was 41.30%, and its corresponding energy was 35 MeV and fluence was 5×10^{10} ions/cm^2. For all generations of treated *Oenothera speciosa*, the overwintering rates in the first year for M_1 generation is 20.1%, M_2 52.0%, M_3 100%, whereas the average overwintering rate for controls is only 2.5%. In addition, there were no relevance among overwintering rates for M_1 *Oenothera speciosa* treated in four years, so were M_2 *Oenothera speciosa* treated in three years. The results suggested that SII treatment can enhance the cold resistance of *Oenothera speciosa* heritably. The overwintering rates of each generations in various years might correlated with the temperatures in that corresponding years (Duan & Jiang, 2002).

3.2. Regional Tests for Cold Resistant M_2 and M_3 *Oenothera speciosa*

A total of five regional testing places, 3710 m^2, were set up for field planting the M_2 *Oenothera speciosa* between Apr to May in 2012, whereas six places (1220 m^2) for M_3 plants in the Apr to Jun in 2013. Regional tests for the overwintering rates of the cold-resistant *Oenothera speciosa* were conducted, the results of which were listed in **Table 2**.

The average overwintering rates of the *Oenothera speciosa* in five testing places was 84.4% in 2013, whereas the overwintering rate of the eleven testing places reached 100% in 2014, which was probably resulted from the temperature differences between two years. It was necessary to be verified by the meteorological data. The regional tests revealed SII-treated M_2 and M_3 generations of *Oenothera speciosa* had satisfactory landscaping effect, which suggesting that SII method could improve the cold resistance of *Oenothera speciosa* heritably and made it overwintered in Beijing and its suburban areas.

3.3. The Relationship of Temperature and Overwintering Rate

Generally, aboveground parts of cold-resistant *Oenothera speciosa* became withered in last Nov to early Dec, and turned green (the seedlings grew to 3 - 5 cm in height, leaves expanded) in mid-late Apr next years. In this study, to analyze the relationship between air temperature and overwintering rate, we used the monthly average temperatures and monthly accumulated temperatures in Nov, Dec in current years, and Jan, Feb, Mar, Apr in the next years from 2011 to 2014 continuously (**Table 3**).

As **Table 3** shown, between 2011 and 2012, the monthly average temperatures of the six months were 3.6˚C, the monthly average positive temperatures in the four months was −57.3˚C·28~31d, and the overwintering rate is 75.2%.

In 2013, the monthly average temperature was 2.3˚C, and the monthly average positive temperature was −73.1˚C·28~31d. The average temperature was 12.8˚C (<15˚C) in Apr. The overwintering rate was 38.9%, which was the lowest in this study. By contrast, 2014 was the warmest in the four testing years with the monthly average temperatures 5.9˚C, the monthly average positive temperature −21.7˚C·28~31d, and the average temperature 17.4˚C in Apr, the overwintering rate was 93.4% which lead to the highest.

The overwintering rates of cold resistant-*Oenothera speciosa* might be affected by the air temperatures. Specifically, they were able to overwinter smoothly in the −137˚C monthly positive accumulated temperature and turn green when the monthly average temperature reach to 15˚C, according with the growth habit of thermophilous plants which can grow normally in the condition with 15˚C air temperature (Duan & Jiang, 2002).

3.4. The Effect of Low Temperature on Relative Electrical Conductivity, Malondialdehyde (MDA) and Proline Contents

Relative electrical conductivity, Malondialdehyde (MDA) and Proline contents of SII-treated M_1, M_2 and M_3 generations of *Oenothera speciosa* and control were detected in the designed temperatures.

As **Figure 3** shown, with the temperature decreased from 20˚C to 5˚C, the relative electrical conductivities

Table 3. Monthly average temperatures and the monthly negative accumulated temperatures in winters in testing years (˚C ˚C·28~31d).

Month	2011 y-2012 y		2012 y-2013 y		2013 y-2014 y	
	average temperature	negative accumulated temperature	average temperature	negative accumulated temperature	average temperature	negative accumulated temperature
11	6.4	−0.1	4.1	−3.0	6.4	−1.9
12	−1.3	−63.7	−4.1	−127.5	0.7	−31.3
1	−3.5	−137.2	−3.9	−120.9	0.4	−20.3
2	−1.4	−28.3	−0.6	−41.0	0.2	−33.4
3	5.8	–	6.5	–	10.3	–
4	15.7	–	12.8	–	17.4	–
Average	3.6	−57.3	2.5	−73.1	5.9	−21.7
Overwintering rate (%)	75.2		38.9		93.4	

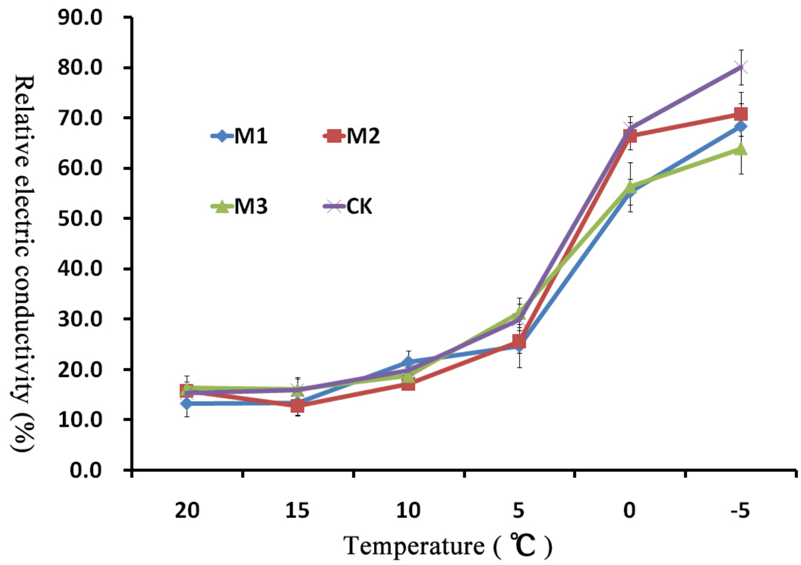

Figure 3. Comparative analysis of relative electrical conductivities of each generation of SII-treated *Oenothera speciosa* and control in different temperatures.

were increasing slowly. However, when the temperature reached to 0˚C, the relative electric conductivity were rising steeply, although no significances among differences of each sample were observed (**Figure 3**). The some differences between each generation of SII-treated *Oenothera speciosa* and control were detected when the temperature declined for 0˚C to −5˚C. The comparisons of the relative electrical conductivities among the samples at −5˚C were described as CK > M_2 > M_1 > M_3 (**Figure 3** and **Table 4**).

As **Figure 4** shown, with the temperature declining, the MDA contents showed a curve-pattern change, that is, no distinct changes of MDA contents was observed from 20˚C to 5˚C; the increasing trends of MDA contents started as the temperature declining from 5˚C to 0˚C; rose steeply from 0˚C to −5˚C; decreased significantly from −5˚C to −10˚C and increased slightly from −10˚C to −20˚C. In this changing course, the some differences of MDA contents between each generation of SII-treated *Oenothera speciosa* and control were only detected during the temperature change from 0˚C to −5˚C. The comparison of the MDA contents among samples at −5˚C were described as CK > M_1 > M_2 > M_3 (**Figure 4** and **Table 4**).

As the temperature declining, the Proline contents showed a W-shape changing pattern (**Figure 5**). The Proline contents were some decreased at 20˚C to 15˚C, smoothly changed at 15˚C to 5˚C, distinctly increased at 5˚C to −5˚C, dramatically declined at −5˚C to −15˚C and slightly increased at −15˚C to −20˚C. During the Proline changing process, the some differences between each generation of SII-treated *Oenothera speciosa* and control were only detected at the stage of 15˚C to 5˚C. The comparison of the Proline contents among samples at −5˚C were described as M_3 > M_2 > M_1 > CK (**Figure 5** and **Table 4**).

Because the distinct observations of relative electrical conductivities, MDA contents, proline contents were occurred in −5˚C, as further analyzed the significances of the differences among each generation of SII-treated *Oenothera speciosa* and control comparatively (**Table 4**).

For the relative electrical conductivities, there were some differences between the generations of M_1, M_2, M_3 and the CK, but no significant difference. For MDA contents, various generations and CK represented some differences, as the same with relative electrical conductivities there was no significant difference. For the Proline contents, the same results were obtained. In addition, for all three physiological indexes, the differences among generations were not significantly (**Table 4**).

The previous studies revealed that low temperature stress can damage the cell membrane system and increase membrane permeability, by which resulted in intracellular electrolyte leakage and relative electrical conductivity increasing (Zheng, 2006). In this study, the results that the relative electrical conductivities of M_1 and M_3 generations of SII-treated *Oenothera speciosa* were lower than CK in −5˚C, showed the improvement of cold resistance of SII-treated *Oenothera speciosa*. In addition, the accumulation of membrane lipid peroxide such as

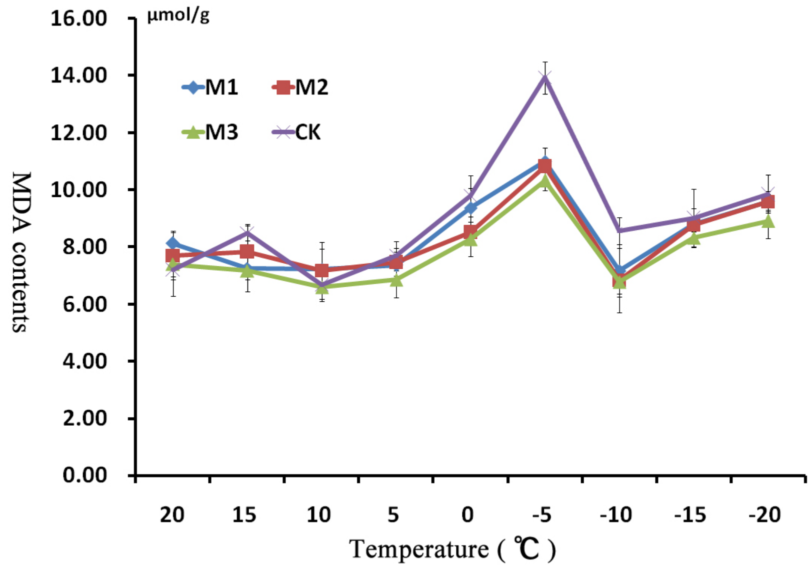

Figure 4. Comparative analysis of MDA contents of each generation of SSI-treated *Oenothera speciosa* and control in different temperatures.

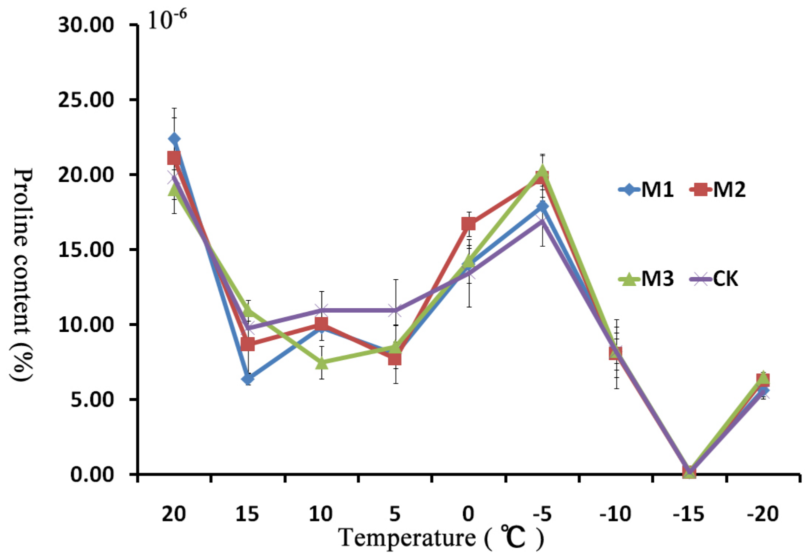

Figure 5. Comparative analysis of Proline contents of each generation of SSI-treated *Oenothera speciosa* and control in different temperatures.

MDA revealed the degrees of damage caused by abiotic stresses. The more accumulation of MDA, the worse membrane system were damaged (Gao et al., 2010; Wang, B.S., 2010). Therefore, the MDA contents of SII-treated *Oenothera speciosa* were less than that in control, suggesting their enhancement in cold tolerance. Furthermore, proline was an important osmoregulation substance to protect plants from ruining by abiotic stresses. However, in this study, only proline contents in M_3 *Oenothera speciosa* had some difference and CK, which might the *Oenothera speciosa* is wild species. It was consistent with the study that some mutants and undomesticated species are resistant to low temperature, although they contain relative low level of proline (Wang, B.S., 2010). Previous researches reveal that low temperature can accelerate the accumulations of proline contents by several or hundred times among various plant species or cultivars under low temperature stress, and the resistance is enhanced as the increasing of proline contents (Jian & Wang, 2009). Low temperature treatment for 4 species of Sedum with the decrease of the temperature, the relative electrical conductivity, MDA content and

Table 4. Comparative analysis of relative electrical conductivities, MDA contents, Proline percent contents of each generation of SSI-treated *Oenothera speciosa* and control in −5˚C.

Samples	Relative electrical conductivity (%)	MDA (μmol/gFW)	Proline (10^{-6} %)
M_1	68.33 ± 4.54b	11.03 ± 0.15b	17.90 ± 1.11ab
M_2	72.70 ± 2.42ab	10.81 ± 0.65b	19.80 ± 1.40ab
M_3	66.07 ± 2.97b	10.33 ± 0.25b	20.33 ± 1.06a
CK	80.07 ± 3.45a	13.95 ± 0.40a	16.88 ± 1.63b
p	0.005	0.000	0.028

Note: Values are mean ±*SE*, different letters within the same column indicate mean values statistically different at $p < 0.05$ as determined by LSD test.

proline content were increasing, there was significant difference below −9˚C to 15˚C, and no significant difference above −6˚C for relative electrical conductivity; but the there were significant difference between −6˚C and −12˚C for MDA; It was no significant difference for proline content (Wang, L.J., 2010). Using low temperature stress for 8 cashew adult blade and 5 cashew family seedlings that results showed the physiological and biochemical indices of cold resistance had complex relationship. The relative electrical conductivity, the content of MDA and proline can be used as cold resistance evaluation index (Wan, 2011). In this study the determination results are also complex. In the future more study will be conduct.

4. Conclusion

In the study, using the SII method, we obtained the heritable cold-resistant *Oenothera speciosa* which can overwinter in open ground in Beijing. The improved *Oenothera speciosa* represented as perennial ornamental groundcover plant with many positive characters such as higher ground coverage, longer florescence, freely flowering, tolerance to low temperature, water-deficit and infertile, and resistant to diseases and pests. It could be applied in landscaping and enriched the green ground cover plants in Beijing.

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