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Comparative Study for Cold acclimation physiological Indicators of *Forsythia mandshurica* Uyeki and *Forsythia viridissima* Indl.

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Abstract: The experiment for the evaluation of comparative effects of cold acclimation for physiological indicators in *Forsythia* species was conducted at Shenyang Agricultural University, China during 2010. Two species of *Forsythia* were compared for cold hardiness comparison i.e *Forsythia mandshurica* Uyeki *and Forsythia viridissima* Indl. The temperature levels were 4, 8, 12 and 22°C. Cold hardiness physiological indicators compared were MDA contents, protein contents, relative leakage of electrolytes, proline contents, POD activities, SOD activities and soluble sugar contents. It was noted that *Forsythia mandshurica* species had higher MDA, protein contents, lower POD activities, SOD activities and sugar contents at lowest (4°C). For relative leakage of electrolyte and proline contents both the species showed almost same pattern as indicators against cold stress. By comparison of physiological indicators under cold hardiness, it was concluded that species *Forsythia mandshurica* was better in cold resistance than *Forsythia viridissima.*

Key words: *Forsythia* · Cold acclimation · Physiological indicators · Antioxidant enzymes

INTRODUCTION

Cold is a major environmental limitation to crop productivity and to the distribution of wild species. It ahs been estimate an annual expenditure of $100 million to minimise frost damage to crops and annual losses of $10-100 million or higher from freezing damage [1]. In addition there is the economic effect of the limitation to crop distribution posed by low positive temperatures (149). Survival of plants at freezing temperatures is dependent on their ability to cold acclimate in response to environmental stimuli such as short-days and low temperatures [2, 3]. Chilling stress is one of the main factors affecting yield and quality of crops. The major injury caused by low temperature (chilling) stress to plants is related to oxidative damage at cellular level [4-6]. Plant species in cold climates have evolved adaptations such as dormancy, rapid acclimation and maintenance of high cold hardiness throughout winter singly or in combination [7, 8].

Forsythia originated in China. They can take full sun or light shade, doing best in well drained soils with supplementary watering during prolonged dry spells. First introduced into Europe in the early 1800's, there are seven species of Forsythia and five wild variants and currently 41 cultivars are listed, all of which are deciduous shrubs with yellow flowers appearing in the spring. Seeds of the Forsythia have been used for medicinal purposes having anti-bacterial, anti-fungal and other properties [9]. *Forsythia suspensa* is a deciduous shrub, native to East Asia, which Xowers in spring before the leaves appear. Recrystallisation inhibition activity was identified in *F. suspensa* by Doucet *et al.* [10].

Exposure of plants to low, but above zero, temperatures has long been known to enhance their subsequent tolerance of exposure to sub-zero temperatures. This process, known as cold acclimation, has been extensively studied, because analysis of the specific alterations associated with cold acclimation could reveal the molecular basis of freezing tolerance in plants

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[11, 12]. The *Forsythia* genus is an excellent system in which to test the hypothesis that the size of dormant flowers is associated with the presence of the deep supercooling characteristic, have been described by Flinn [13].

Physiological, structural and biochemical analysis identifies several consistent features of cold-acclimation which are thought to have an important role in cold-tolerance; well-known examples are solute accumulation and changes in membrane lipid composition [14, 15]. However, temperature impinges on all aspects of plant metabolism and physiology and thus there are probably very many important changes in response to cold, some as yet unknown. Higher plants have developed an oxygen-scavenging system which consists of some antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD) and catalase [16]. In the last 15 years, molecular analysis has provided a different approach to analysing acclimation and adaptation to cold with the potential to extend and more critically test old ideas and to add new ones [17].

In the view of above studies, the main objective to carry out this study was to compare the Cold Acclimation Physiological Indicators of *Forsythia mandshurica* Uyeki and Forsythia *viridissima* Indl. by undertaking many physiological attributes.

MATERIALS AND METHODS

Sample leaves of Forsythia species were collected from 30th September to 16th October 2010 in Shenyang Agricultural University, China (41°82'N, 123°56'E). Samples were collected at different temperatures 4, 8, 12 and 22°C and the leaves were wrapped by aluminum foil in liquid nitrogen. Samples were placed in -70°C refrigerator for study of cold hardiness indicators as:

- MDA contents
- Protein contents
- Relative leakage of electrolytes
- Proline contents
- POD activities
- Soluble sugar contents
- SOD activities

Determination of Malondialdehyde (MDA) Contents: Lipid peroxidation was determined by estimating the malondialdehyde (MDA). Fresh samples mixed with 5 mL phosphate buffer (pH 7.8) were crushed into homogenate in a mortar. The homogenate was centrifuged at 10,000 g for 20 min at 4°C, using the supernate for MDA determination. A mixture of 1 mL extracts (MDA) + 2 mL 0.6% thiobarbituric acid (TBA) (0.6 g TBA + 1 M NaOH + 10% trichloroacetic acid complete to 100 mL) was produced, boiled for 15 min, cooled and centrifuged for 10 min (4000 rpm). The concentration of MDA was calculated from the absorbance at 600, 532 and 450 nm.

Determination of Protein Contents: Soluble protein concentration was measured by the Bradford [18] method with Bovine Serum Albumin (BSA) as the standard.

Relative Leakage of Electrolytes: Samples were boiled for 7 min to kill the tissues, then cooled and brought back to the initial volume by adding distilled water and held at 20°C for 24 hrs. Then electrical conductivity was measured. According to the values of electrical conductivity both percentage of electrolytes and index of injury were calculated as:

$$\text{Electrolytes (\%)} = \frac{\text{EC (}\mu\text{ mohs) before boiling}}{\text{EC (}\mu\text{ mohs) after boiling}} \times 100$$

The index of injury (I_t)

$$I_t = 100\,(L_t - L_0)/(L_k - L_0)$$

Where:

I_t = Index of injury resulting from exposure to temperature 4°C or -4°C.

L_t = Electrical conductivity (EC) from sample exposed to temperature 4°C or -4°C.

L_0 = Electrical conductivity (EC) from sample kept at room temperature.

L_k = Electrical conductivity (EC) from sample exposed to temperature 4°C or -4°C and then kept killed.

Determination of Proline Content: Determination of free proline content was done according to Bates *et al.* [19]. Leaf materials (0.5 g) were homogenized in 3% (w/v) sulfosalicyclic acid and homogenate filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated at 100°C for 1 h in a water bath. The reaction was then stopped by using an ice bath. The mixture was extracted with toluene and the absorbance of the fraction with toluene extracted from the liquid phase was read at 520 nm. Proline concentration was determined using a calibration curve and expressed as μg proline g^{-1} FW.

POD Activity Assay: Guaiacol Peroxidase (POD) activity was measured with guaiacol as the substrate in a total volume of 3 mL according to Zhang [20]. The reaction mixture consisted of 25 mmol L^{-1} phosphate buffer (pH 7.0), 1.5% guaiacol, 0.4% H_2O_2 and 0.2 mL of enzyme extract. Increase in the absorbance due to oxidation of guaiacol (E=25.5mmol L^{-1} cm^{-1}) was measured at 470 nm. Enzyme activity was calculated in terms of µmol of guaiacol oxidized g^{-1} FW min^{-1} at (25± 2)°C.

Determination of Soluble Sugar Contents: Soluble sugar content was measured following the method described by Yemm and Willis [21]. Leaf materials were soaked in 25 mL distilled water. The solution was boiled (100°C) for 30 min to extract soluble sugar and centrifuged under 4000 rpm for 10 min. The extracts were decanted and the residue was re-extracted for twice more, with extracts being completed to 50 mL. In all, 0.1 mL extracts and 3 mL anthrone reagent (0.15 g anthrone+84 mL oil of vitriol+16 mL H_2O) were mixed and the absorbance of the mixture was recorded at 620 nm. The content of soluble sugar was calculated from a standard curve of glucose at 620 nm by colorimetry.

SOD activity Assay: SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to Rao and Sresty [22]. The reaction mixture was 3 mL, which contained 50 mmol L^{-1} phosphate buffer (pH 7.8), 13 mmol L^{-1} methionine, 75 µmol L^{-1} NBT, 2 µmol L^{-1} riboflavin, 0.1 mmol L^{-1} EDTA and 0.1 mL of enzyme extract. Reaction was started by adding 2 µmol L^{-1} riboflavin and placing the reaction tubes under 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme extract served as a control. Reaction was stopped by switching off the lamp.The photoreduction of NBT was measured at 560 nm and one unit of SOD was defined as being present in the volume of extract that caused inhibition of the photoreduction of NBT by 50%.

RESULTS AND DISCUSSION

During the comparison for cold acclimation physiological indicators between two species of *Forsythia* following results were obtained.

Malondialdehyde (MDA) Contents: Results for MDA contents are presented in (Fig. 1). It was noted that *Forsythia mandshurica* species had higher MDA contents at lowest (4°C) and highest temperature (22°C). On the other hand between 8-12°C, *Forsythia viridissima* had lower MDA contents and *Forsythia manshurica* had higher MDA contents. Anderson *et al.* [23] reported that the harm caused by chilling stress was, in part, due to membrane lipid peroxidation. The MDA is an indicator of lipid peroxidation and links to peroxidation of polyunsaturated fatty acids in the membranes thereby releasing free radicals [24].

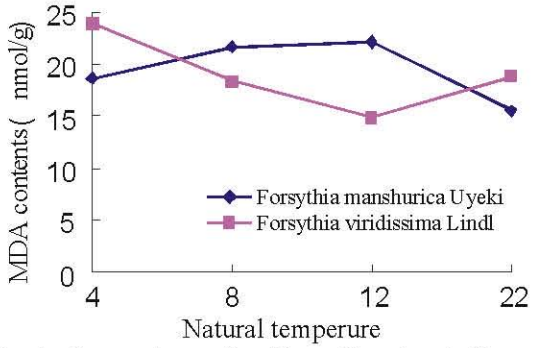

Fig. 1: Comparison of cold acclimation indicators of MAD in two species of *Forsythia*

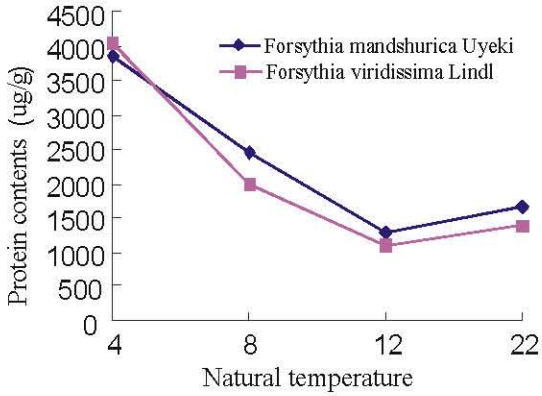

Fig. 2: Comparison of cold acclimation indicators of protein contents in two species of *Forsythia*

Protein Contents: Protein contents in both species of *Forsythia* were increased at lower temperature hardiness (Fig. 2). Minimum protein contents were noted at 12°C in both species of *Forsythia.* At lowest temperature (4°C) there were higher protein contents in *Forsythia viridissima*. These results are in accordance of with Hwei-hwang and Li [25]. They noted that soluble protein contents were increased in both *Solanum acead* and *Solanum commersonii.* Net increases of the soluble proteins were positively and significantly correlated with net increases of frost hardiness in *S. acaule* and *S. commersonii.*

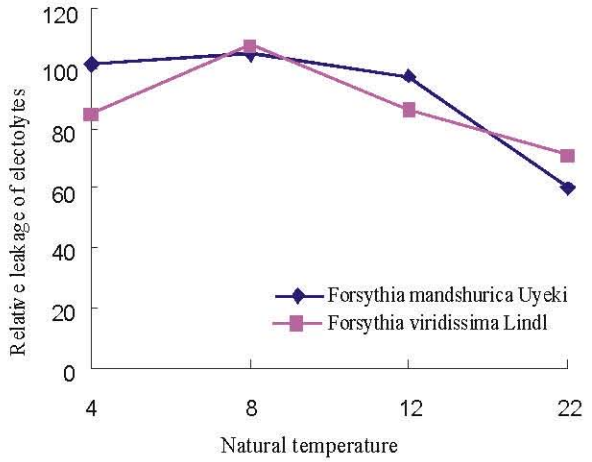

Fig. 3: Comparison of cold acclimation indicators of Relative leakage of electrolytes in two species of *Forsythia*

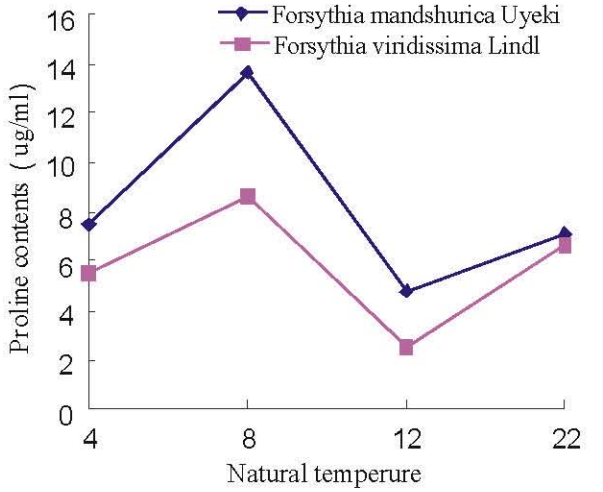

Fig. 4: Comparison of cold acclimation indicators of proline contents in two species of *Forsythia*

Relative Leakage of Electrolytes: The electrolyte leakage test was well suited for measuring freeze-induced damage as it is based on alterations in cell membranes, i.e. in the locus of initial injury. Furthermore, the test is fairly simple and rapid, yields quantitative data and requires only small amounts of plant material. However, certain concerns limit the validity of the technique. Data for relative leakage of electrolytes have been shown in (Fig. 3). At lower temperature (8°C) in *Forsythia viridissima* had highest ration of electrolyte leakage and at highest temperature (22°C) in *Forsythia mandshurica* had lowest ration of electrolyte leakage. These results are in accordance with Abd El-Moniem *et al*. [26].

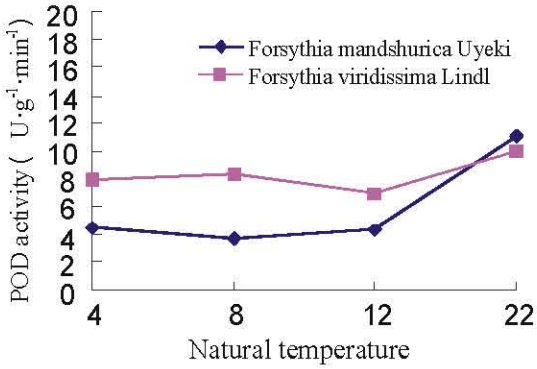

Fig. 5: Comparison of cold acclimation indicators of POD activities in two species of *Forsythia*

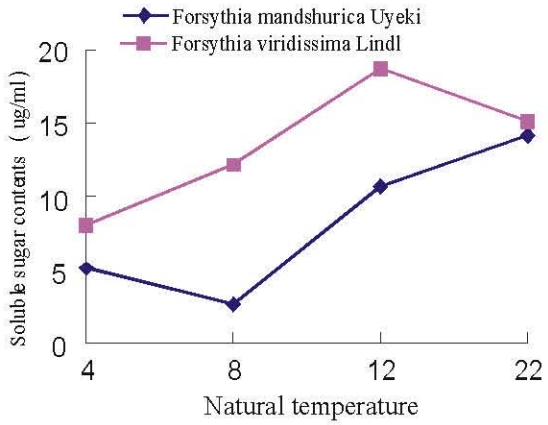

Fig. 6: Comparison of cold acclimation indicators of soluble sugar contents in two species of *Forsythia*

Proline Contents: Data for proline contents has been plotted in (Fig. 4). It was observed that proline contents were higher in *Forsythia mandshurica* than *Forsythia viridissima*. Maximum proline contents were noted at 8°C and lowest at 12°C in both species. At lower temperature (8°C) proline contents was increased in *Forsythia mandshurica*, which reached a higher value in comparison to the resistant one. Pattern on proline contents variations was same in both species. Among the several protection mechanisms against low temperatures adaptability at high altitude increased proline content acting as osmoregulant has been reported [27].

Guaiacol Peroxidase (POD) Activities: By comparison of POD activities between two species, it was observed that it decreased with low temperature stress (Fig. 5). Pattern of both the species were same under variations of temperatures. The researchers generally agree that POD activity is related to the degree of cold tolerance of plants.

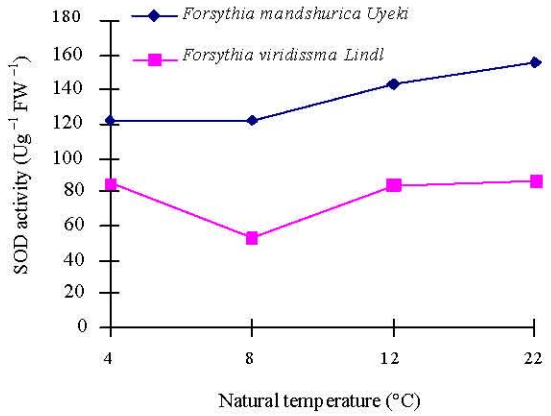

Fig. 7: Comparison of cold acclimation indicators of SOD activities in two species of *Forsythia*

As the activities of POD increase, the cold resistance of plants is stronger [28].

Soluble Sugar Contents: Soluble sugar contents were higher in *Forsythia viridissima* than *Forsythia mandshurica* (Fig. 6). In both the species soluble sugar contents decreased with the decrease in temperate. Minimum soluble sugar contents were noted at 4°C. Sucrose and other sugars play a central role in cold stress as signalling molecules that modulate the physiology, metabolism and development of plants [29, 30].

SOD Activity: SOD activity were higher in *Forsythia mandshurica* than *Forsythia viridissima* (Fig. 7). In *Forsythia mandshurica* SOD activity increased with the increase in temperate. Minimum SOD activity were showed at 8°C in *Forsythia viridissima*. SOD dismutes active oxygen radical (O_2) into H_2O_2 and plays a key role in cellular defense against reactive oxygen species [31, 32].

In northern areas, low temperature is the major environmental factor limiting the productivity and the geographical distribution of horticultural plants. Adaptation to seasonal changes in temperature is a precondition for woody plant life in temperate and boreal vegetation zones. In this experiment, the physiological responses to different temperature were studied to identify some of the key elements that may be responsible for abiotic stress tolerance in two species of *Forsythia*. The concentrations including protective enzymes, oxidative stress products, relative leakage of electrolytes and soluble sugar were analyzed.

For better understanding of chilling stress responses in tobacco (*Nicotiana tabacum*), growth rate and antioxidant enzymes of seedlings in 2 tobacco cultivars at chilling temperature (5°C) were studied [16]. In *Forsythia mandshurica* has higher SOD activities, protein contents and proline contents at low temperature than *Forsythia viridissima*(Fig. 2. Fig. 4. Fig. 7). The researchers generally agree that SOD activities, protein contents and proline contents is related to the degree of cold tolerance of plants.As their amount increase, the cold resistance of plants is stronger. However, the experiment of POD suggests that POD activity has little influence in two species of *Forsythia* under chilling condition (Fig.5).

In previous research, MDA content was lower while more efficient radical scavenging enzymes were observed in plants [33]. Polyunsaturated fatty acids were degraded to produce malondialdehyde (MDA) under low temperature. It was observed that changes in MDA content in a tissue could be a good indicator of the structural integrity of the membranes of plants subjected to chilling stress [34]. MDA contents in *Forsythia mandshurica* was increased under cold condition (4-12 °C), while it was decreased at normal temperature (22 °C), however, *Forsythia viridissima* is counter to *Forsythia mandshurica* (Fig.1).

Study of Flinn [13] showed that the strength of the correlation between *Forsythia* hardiness and pistil size was likely affected by the method used to quantify pistil size in the current study. They research shows that *Forsythia mandshurica* had more cold resistance than *Forsythia viridissima* in *Forsythia* buds affect hardiness. By comparison of physiological indicators under cold hardiness, it was concluded that species *Forsythia mandshurica* was better in cold resistance than *Forsythia viridissima,* which confirms the previous results of Jouve *et al.* obtained with the same and species and reflects their differet ecology [34].

The ultimate survival of woody plants is dependent on not only the maximal capacity of cold hardening, but also on the timing and rate of both cold acclimation and deacclimation, the stability of cold hardiness and the ability to reacclimate after unseasonably warm periods [35-36].Therefore, the ability to estimate the degree of cold hardiness in plants is of great value for both basic and applied studies.

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