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LOW TEMPERATURE STRESS INDUCED CHANGES IN THE PHENOLIC CONTENTS AND ITS REGULATORY ENZYMES IN SAL SEEDLINGS

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SUMMARY

Changes in growth pattern and phenol levels were studied in sal seedlings in response to low temperature (9-14.1^{0}C, night temp.) in the field and under greenhouse conditions (Temp. 30-32^{0}C, RH 70-76%, Light intensity 33523 Lux). Constant exposure of field grown seedlings to low-temperature (during November to March) resulted in injury. Nearly 80-86% seedlings exhibited desiccated, necrotic leaves and shoots along with severe reduction in dry mass. Abrupt increase in the phenol levels (1.8-2.2 folds) was discernible both in the leaves and shoots with the drop in air temperature below 14.1^{0}C. Overproduction of phenols in aerial parts during this period commensurate with both enhanced activity (1.5 and 1.7 folds in the leaves and shoots respectively) of its synthesizing enzyme L-phenylalanine ammonia lyase (PAL) and simultaneous reduction (8 and 13 folds in the leaves and shoots, respectively) in its degrading enzyme polyphenol oxidase (PPO). As a result, accumulation of excess levels of phenolics culminates into irreversible injury in aerial parts of sensitive sal seedling in response to low-temperature that is one of the causes of mass scale mortality in these seedlings.

Key words: **Low temperature injury, L-phenylalanine ammonia lyase, phenols, polyphenol oxidase, *Shorea robusta***

INTRODUCTION

Sal forest comprises of 14.1% (largest forest area) of the total forest cover in India. Like several tropical and sub-tropical plants, aerial growth in most of the young sal seedlings was arrested in the forest sites during the winter season (Keshavkant and Naithani 2005). Constant exposure of air temperature critically ranging between 9-14.1^{0}C (low-temperature) resulted in mass scale mortality in sal seedlings (Keshavkant and Naithani 2005). The most common symptoms of low-temperature induced injury include conspicuous discoloration leading to necrosis of leaves and shoot tissues (Lafuente *et al*. 2004). Temperature dependent phase transition in the cellular membrane has been postulated as the primary response of sensitive species to low-temperature (Uemura *et al*. 2003) and these are thought to induce accumulation of toxic metabolites thereby leading to cellular damage (Kirakosyan *et al*. 2003).

The synthesis of phenolic compounds is often enhanced in plant tissues under stresses such as mechanical damage (Reyes and Cisneros-Zevalloss 2003) or infection by microorganisms (Seo *et al*. 2003). Similarly, increased amounts of polyphenols were considered as a characteristic feature of secondary metabolism to low-temperatures in leaves of *Crataegus monogyna* and *Triticum aestivum* (Kirakosyan *et al*. 2003, Olenichenko and Zagoskina 2005). Symptoms such as brown pitting, necrosis, deterioration of mitochondrial activity and cell damage have been associated with increased deposition of phenolic compounds (Fukumoto *et al*. 2002).

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L-phenylalanine ammonia lyase (PAL) is a key enzyme, in the biosynthetic pathway of polyphenols in plants (Sanchez-Ballesta *et al*. 2000, Lafuente *et al*. 2004). Much attention has been focused on PAL as this enzyme showed striking changes in activity in response to a wide variety of external factors such as light, disease, wounding and low-temperature (Sanchez-Ballesta *et al*. 2000, Reyes and Cisneros-Zevalloss 2003). PAL activity is commonly detected around the necrotic zones of Fortune fruits (cross pollinated species of *Citrus clementina* and *Citrus reticulata*) stored under low-temperatures (Sanchez-Ballesta *et al*. 2000). Enhanced PAL activity has been suggested both due to *de-novo* synthesis as well as release of PAL from inactive enzyme inhibitor complex (Engelsma 1970). Accumulation of polyphenols in the plants is controlled by polyphenol oxidase (PPO), also known as phenolase, through the oxidation of o-diphenols to quinones as well as hydroxylation of monophenols (Ose *et al*. 1999). PPO is widely considered as a plastid enzyme although it was reported in the cytoplasm of fruit tissues during ripening followed by senescence (Flurkey and Jen 1978). Reduced activity of PPO was recorded during low-temperature stress in *Ipomoea aquatica* (Ose *et al*. 1999). The aim of this study was to understand the changes in the phenol content during low-temperature injury in sal seedlings. Activities of PAL and PPO were also monitored to establish their role in response to low-temperature induced stress and their contribution in the homeostasis of phenolics in sal seedlings.

MATERIALS AND METHODS

Seed collection and nursery raising: Mature sal seeds were collected from the Gariyabandh forest reserve, 91 km to North-East of Raipur (20^{o}38'N latitude, 82^{o}04'E longitude and 306 masl). The freshly collected seeds were germinated between two wet jute bags. The two days old germinants with 10-15 mm radicle were sown in polybags (15x22cm) containing mixture of black soil, sand and farm yard manure (2:1:1,V:V:V). The seedlings were watered twice a day. The first sampling was made after 7 days of emergence of first two leaves. Subsequently seedlings were sampled after every 30 days, from the day of emergence of the first two leaves. Nursery of sal seedlings was maintained in the Botanical Garden of School of Life Sciences, Pt. Ravishankar Shukla University, Raipur. Four months after seedlings emergence, *i.e.* in October, the seedlings were randomly divided into two groups of 4000 seedlings each, of which one group was shifted to greenhouse (Temp. 30-32^{o}C, RH 70-76%, Light 33523 Lux). The irradiance of light (day time only) measured in the field and greenhouse showed a difference of 80-100 Lux throughout the period of analysis. All biochemical analyses were performed in five replicates and repeated twice.

Meteorological data: Data on air temperature for field condition was obtained from the Department of Physics and Agrometeorology, Indira Gandhi Agriculture University, Raipur [5 Km away from Pt. Ravishankar Shukla University (air distance)], whereas the air temperature and relative humidity data for greenhouse were recorded by employing a thermohygrometer (Table 1).

Percentage of injured seedlings: The percentage of injured seedlings was recorded every month in field and greenhouse as described elsewhere (Keshavkant and Naithani 2005). The important visual observations of injured seedlings were surface pitting, browning and necrosis of leaves subsequently followed by necrotic shoot tip of seedlings.

Growth analysis: Ten seedlings were randomly selected and harvested for biomass determination. Dry mass of the leaves and shoot were recorded after oven drying at 85^{o}C for 72hrs (Keshavkant and Naithani 2005).

Extraction and estimation of phenols: Tissue extracts were prepared by homogenizing 1g of plant tissue with sterilized silica and 5 ml of 80% ethyl alcohol in borate buffer (0.2M, pH 7.6). The homogenates were centrifuged at 8945xg for 5 min at room temperature and the supernatant was used to quantify phenols. Total phenol was estimated following the method of Swain and Hills (1959). The reaction mixture contained 20µl phenol extract, 880µl distilled water, 100µl Folin-Ciocalteu reagent (1N) and 2000µl saturated solution of sodium carbonate. The absorbance was measured at 660nm. Chlorogenic acid was used as standard and expressed as mg g^{-1} fm of the tissue. Monophenol was estimated

as described elsewhere (Keshavkant 2000) using hydroxyl benzene as standard. The reaction mixture consisted of 100µl phenol extract, 400µl distilled water, 400µl sodium hydroxide (0.5N), 500µl 4-amino antipyrene (0.6%), 600µl sodium bicarbonate (9.5M) and 500µl potassium ferrocyanide (2.4%). Absorbance was measured at 520nm and expressed as mg g^{-1} fm of leaf and shoot tissue. Diphenol was determined following Mahadevan (1975). The assay mixture comprised of 50µl phenol extract, 450µl distilled water, 1000µl hydrochloric acid (0.5N), 500µl Arnows reagent (10% sodium nitrite and 10% sodium molybdate in 100ml of distilled water) and 1000µl sodium hydroxide (1N). Absorbance was measured at 525nm. Catechol was used as a standard and expressed as mg g^{-1} fm of the plant tissue.

Determination of enzyme activities: Leaf and shoot (2 g) samples were homogenized with sterilized silica in 10 ml of chilled borate buffer (0.2M, pH 7.4). The homogenates were then centrifuged at 35780xg for 20min at 4^{o}C and the supernatant was used to estimate enzyme activity. L-Phenylalanine ammonia lyase assay was carried out following the method of Rosler *et al*. (1997). The assay mixture comprised of 2094µl borate buffer (0.1M, pH 8.8), 50µl L-phenylalanine (10mg/ml) and 10µl enzyme extract. The absorbance was recorded at 290nm and expressed as A_{290} min^{-1} g^{-1} fm of the leaves and shoots. Polyphenol oxidase activity was determined by the method of Yamaguchi *et al*. (1970). The assay mixture consisted of 2900µl of 0.01M catechol (one day old) with 0.01M proline in phosphate buffer (0.01M, pH 6.5) and 100µl of enzyme extract. The absorbance was measured at 525nm and expressed as A_{525} min^{-1} g^{-1} fm of the plant tissue.

Statistical Analysis: One-way ANOVA and linear correlation analyses were performed using COSTAT for showing significant difference between the field and greenhouse sal seedlings. The significant difference is expressed as (*) and (**) respectively, whereas non-significant difference is expressed as NS.

RESULTS

Mortality in sal seedlings was promoted from the month of September to March (Table 1). Maximum mortality (24%) was recorded in the month of December followed by January (22%). The total mortality of seedlings recorded in the field condition (86%) was very high compared to the greenhouse (20%) (Table 1). Dry mass of leaf and shoot demonstrated seasonal variation. One week seedlings (June) showed 0.048 and 0.027g dry mass of leaves and shoots respectively. It increased rapidly (12-18 fold) for another four months (up to October) (Table 2). The reduction in field temperature from November-March resulted in rapid loss of dry mass of leaves and shoots to almost 2.5-3.3 fold (Table 2). On the other hand, the four month seedlings shifted to greenhouse (in October) grew rapidly during November–March and recorded significantly higher accumulation of dry mass both in leaf (1.9 fold) and shoot (2.2 fold) (Table 2).

Table 1. Percentage of injured sal seedlings and minimum air temperature in field and greenhouse conditions. Percentage injury was recorded in the sample population of 100 seedlings in each test conditions and values presented are mean ±SD of 10 replicates. Data presented for minimum air temperature are mean ±SD of 4 independent determinants. *Values are significantly different from the field condition at *P = 0.05* level.

Months	% Injured seedlings		Temperature (minimum^{o}C)	
	Field	Greenhouse	Field	Greenhouse
June	Nil		28.0±0.02	
July	Nil		25.4±0.03	
Aug.	Nil		24.9±0.05	
Sep.	05±0.12		23.6±0.14	
Oct.	09±0.16	Shifted	22.2±0.16	
Nov.	11±0.09	08±0.12*	14.1±0.03	30.0±0.05*
Dec.	24±0.21	06±0.07*	10.8±0.05	32.0±0.09*
Jan.	22±0.12	04±0.21*	09.0±0.12	30.5±0.10*
Feb.	11±0.06	02±0.11*	10.7±0.06	31.5±0.11*
Mar.	04±0.08	Nil	14.0±0.10	31.2±0.08*

Phenol: Accumulation of total phenol (TP) was observed in response to low-temperature in aerial parts of sal seedlings. A gradual rise in TP levels was seen in the leaves (110.84-191.51 mg g^{-1} fm) and shoots

Table 2. Changes in dry mass of leaf and shoot of field and greenhouse grown sal seedlings during 9 months. Data presented are cumulative mean of 10 independent determinants ±SD. *Values are significantly different from the field condition at *P = 0.05* level.

Months	Leaf (g seedling^{-1})		Shoot (g seedling^{-1})	
	Field	Greenhouse	Field	Greenhouse
June	0.048±0.03		0.027±0.01	
July	0.244±0.02		0.149±0.02	
Aug.	0.431±0.01		0.277±0.03	
Sep.	0.546±0.06		0.395±0.02	
Oct.	0.574±0.04		0.491±0.02	
Nov.	0.509±0.03	0.588±0.09*	0.429±0.04	0.495±0.03*
Dec.	0.420±0.05	0.680±0.05*	0.361±0.05	0.554±0.06*
Jan.	0.368±0.02	0.734±0.05*	0.317±0.06	0.607±0.03*
Feb.	0.275±0.03	0.969±0.02*	0.246±0.11	0.953±0.08*
Mar.	0.173±0.02	1.105±0.04*	0.197±0.03	1.105±0.06*

(101.31-145.21 mg g^{-1} fm) of one week to 4 month seedlings (Fig. 1a). Accumulation of TP was slow during November to March in the aerial parts of seedlings that were shifted to greenhouse (Fig. 1a). In contrast, a significant rise (1.8 fold) was discernible in the levels of TP in leaves and shoots of field grown seedlings that were exposed to low-temperature during November to March (Fig. 1a).

The monophenol (MP) was higher in the leaves than that of the shoot of sal seedlings, throughout the experimental period (Fig. 1b). Relatively low levels of MP were recorded in leaf and shoot of one week (16.33 and 13.45mg g^{-1} fm, respectively) and 4 month seedlings (24.65 and 18.06mg g^{-1} fm, respectively) (Fig. 1b). Later on, with the fall of air temperature (in field), an abrupt increase was discernible in the aerial parts of 5 month (in November) seedlings. The highest levels were recorded in highly desiccated leaves (44.21mg g^{-1} fm) and shoot (34.40mg g^{-1} fm) of 9 month seedlings (Fig. 1b). On the other hand, the aerial parts of the greenhouse seedlings displayed significantly low rates of MP accumulation (25.09 and 20.58mg g^{-1} fm in leaves

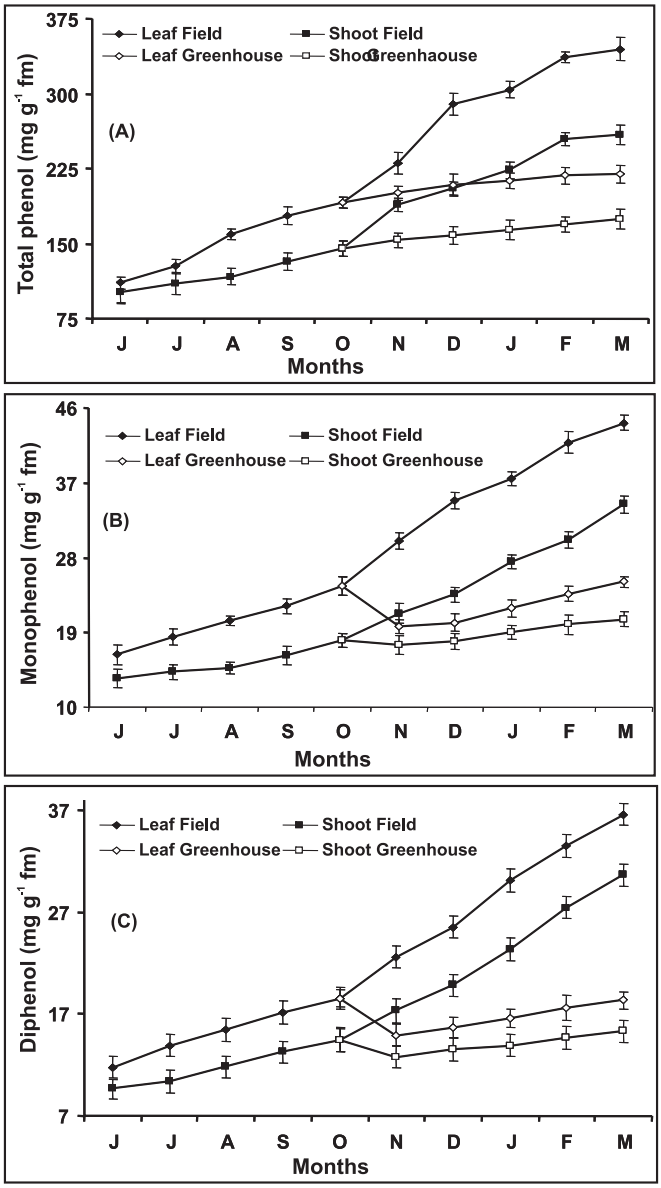

Fig. 1. Changes in total phenol (1a), monophenol (1b) and diphenol (1c) in the leaves and shoots of sal seedlings during nine-months under field and greenhouse conditions. Each point is the mean ±SD of 5 replicates.

and shoot respectively). Diphenol (DP) accumulated slowly in aerial parts of sal seedlings from June (leaf, 11.79 and shoot, 9.68mg g^{-1} fm) to October (leaf, 18.53 and shoot, 14.47mg g^{-1} fm) (Fig. 1c). Comparatively, the DP content was higher in leaves than the shoots. In November, with the reduction in air temperature to 14.1^{o}C, the levels of DP increased sharply registering 2

- 2.2 fold rise in leaves and shoot respectively by March in 9 month seedlings whereas, significantly less accumulation of DP was observed in leaves and shoots of 9 month greenhouse seedlings (Fig. 1c).

L-phenylalanine ammonia lyase: Gradual increase in PAL activity was recorded in one week to 4 month seedlings (Fig. 2a). Later, with the reduction in air temperature (in field), equally high activity of PAL was discernible in the aerial parts of 5 month (in November) seedlings. The highest levels were recorded in desiccated shoots and leaves of 9 month seedlings (Fig. 2a). In contrast, applying one-way ANOVA, significantly lower activity of PAL was registered in the leaves and shoots of the greenhouse seedlings during November to March (Fig. 2a).

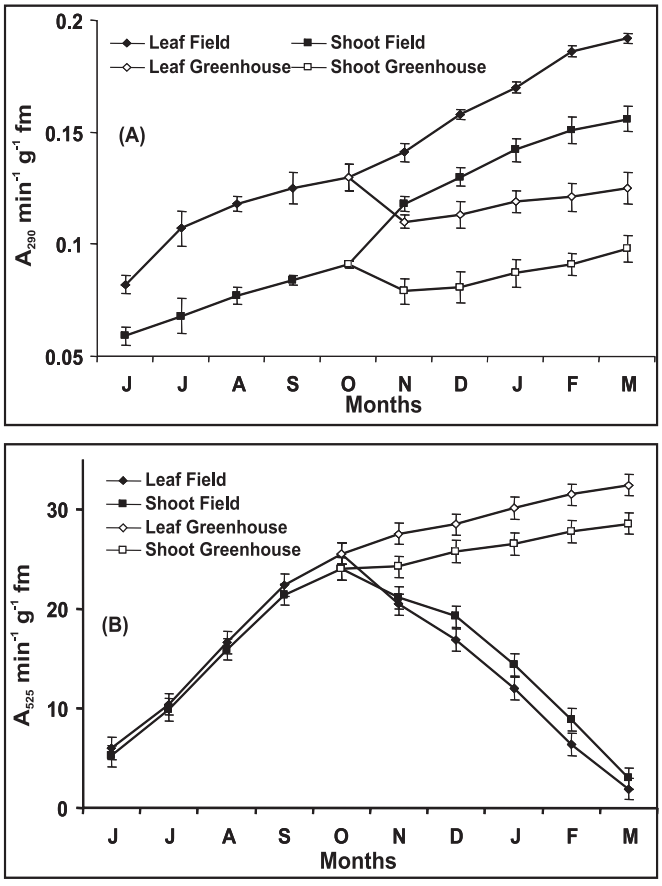

Fig. 2 Changes in L-phenylalanine ammonia lyase (2a) and polyphenol oxidase (2b) activities in leaves and shoots of greenhouse and field grown sal seedlings. Data presented are mean ±SD of 5 individual replicates

Polyphenol oxidase: PPO activity was higher in leaves as compared to shoots of sal seedlings (Fig. 2b). Gradual rise in PPO activity was discernible in the leaves and shoots from one week to 4 month seedlings (Fig. 2b). Later, with the fall of air temperature from November, the PPO activity declined sharply (8-13.3 fold) and reached to minimum by March in shoots and leaves of field grown sal seedlings (Fig. 2b). On the other hand, seedlings growing in the greenhouse recorded significantly high activity of PPO in aerial parts during November to March (Fig. 2b).

DISCUSSION

Sal seedlings exhibited typical periodic growth phenomenon in field condition (Keshavkant and Naithani 2005). One week seedlings showing 0.048 and 0.027g dry mass of leaf and shoot respectively, grew rapidly up to October with 12-18 fold accumulation in dry mass (Table 2). Constant exposure of seedlings to low-temperature (below 14.1^{O}C, night temperature) during November to March resulted in desiccated, brown and necrotic leaves/shoot along with severe loss in dry mass (2.5-3.3 fold) in 80-86% seedlings (Table 1 and 2). In tropical and subtropical plants such as *Zea mays*, *Dracaena sanderana* and *Citrus clementina*, low-temperature induced inhibition in growth along with injury symptoms like brown spotting, discoloration and necrosis is an established indicator of low-temperature sensitivity (Lafuente *et al*. 2001, 2004, Fernandez *et al*. 2003). The greenhouse seedlings in contrast showed 1.9 and 2.2 fold accumulation in dry mass of leaves and shoots respectively during November to March (Table 2).

Phenolic compounds were accumulated (1.8-2.2 fold) in aerial parts of sal seedlings following exposure to low-temperature (Fig. 1a-c). Injured leaf/shoot tissues have been shown to accumulate excessive amounts of phenolics as a result of leakage of these compounds from the vacuoles, thereby causing cellular damage (Fukumoto *et al*. 2002). Appearance of distinct morphological symptoms like brown pitting and necrosis that culminates into cell death in the aerial parts of sal seedlings (Keshavkant and Naithani 2005) may be attributed to excessive amounts of accumulation of various phenolics in response to low-temperature.

PAL activity was promoted substantially in response to low-temperature in aerial parts of sal seedlings (Fig. 2a). Comparatively the PAL activity was significantly higher in leaves than in the shoot tissues (Fig. 2a). Strong positive correlation between PAL activity and corresponding levels of TP in the leaves and shoots of field grown seedlings further approves the direct involvement of PAL enzyme in the biosynthesis of phenols *per se*. Increased synthesis and/or accumulation of various phenolics were positively correlated with *de-novo* synthesis or activation of PAL in several plant tissues (Sanchez-Ballesta *et al*. 2000, Teklemariam and Blake 2004).

Higher levels of phenolics in injured sal seedlings were not only associated with higher PAL activities in these tissues but also coincides well with substantially reduced activity of PPO (Fig. 2b). PPO activity increased during rapid growth (June-October) but reduced sharpely as the sal seedlings were exposed to low temperatures for increasing periods (Fig. 2b). Negligible activities of PPO in severely injured (9-months-old) sal seedlings as compared to vigorously growing seedlings in greenhouse could also be responsible for accumulation of higher levels of phenolics (due to reduced oxidation). Coexistence of significantly high levels of PPO and insignificant activities of PAL resulted in highly reduced levels of phenolics in greenhouse seedlings (Fig. 1a-c). Correlative changes in PPO and PAL activities with low-temperature and phenolic contents have been reported in plant species such as *Musa paradisica* (Nguyen *et al*. 2003), *Castanea henryi* (Xu 2005) and *Prunus persica* (Brandelli and Lopes 2005). The considerable reduction in PPO activity during chilling period (Ose *et al*. 1999) particularly in leaves can be explained by the location of this enzyme in the leaf tissue, the membrane of which is the primary targets during low-temperature induced photoxidation (Keshavkant 2000).

It can be, therefore, concluded that, lower levels of phenolics maintained mainly due to increased PPO and corresponding low levels of PAL are essential indicators of vigorous growth rate in greenhouse seedlings, whereas, reverse was experienced in the field grown seedlings. Thus, it is suggested that the excessive accumulation of phenols might lead to impairment of membrane function leading to cell damage and thereby terminating into mass scale mortality/dieback in field grown sal seedlings.

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