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PIOTR KAROLEWSKI AND PAWEŁ M. PUKACKI

Free proline as an indicator of the sensitivity of Norway spruce (*Picea abies* (L.) Karst.) to low temperature

Abstract

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The content of free proline and water was determined in the needles of two Polish populations of Norway spruce (*Picea abies* (L.) Karst.) held for 24 h at a temperature of 10, 0, −10, −20, −30 and −40°C. Level of the imino acid was determined after 24 h and 108 h following termination of exposition of shoots to the different temperatures. Degree of needle injury was determined by measurement of electrolyte leakage from them. It was found that the level of free proline was a sensitive indicator of the tolerance of the studied populations of spruce to low temperatures. The more sensitive plants reacted at a lower intensity of the stressing factor. The nature of the relationship between the level of free proline in needles of spruce and the low temperatures was similar to that found in earlier studies on the influence of other abiotic stress factors. Depending on the intensity of the stress, there were no changes at first, but later after reaching a certain critical level, there was observable a rapid accumulation and then a decline in the level of this imino acid. The level of water analyzed in needles of spruce and the effluence of ions from them reflects well the level of tissue injury.

Additional key words: abiotic factors, temperature stress, freezing.

Address: P. Karolewski, P.M. Pukacki, Polish Academy of Sciences, Institute of Dendrology, 62-035 Kórnik, Poland.

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INTRODUCTION

The increased tolerance of plants to the action of low temperatures, both in the range of mild temperature stress and at temperatures below 0°C is believed by many authors to be related to the protective role of proline. Borman and Jansson (1980) and Duncan and Widholm (1987) have found that accumulation of proline increases tolerance of plants to the action of low temperature.

Similar conclusions have been reached by Hellergren and Li (1981) and by Swaaij et al. (1985) who have shown that exogenously supplied proline increases tolerance of plants to frost. In experiments no wheat seedlings treated with low temperatures a 50-fold increase in the level of free proline was observed without changes in the water potential of the leaves (Naidu et al. 1991).

The protective function of free proline is primarily associated with its role in the maintaining of a proper water content of tissues. Those who support this theory underline that the more tolerant plants are the ones which in conditions of an osmotic stress are capable of accumulating larger quantities of this imino acid (Levin et al. 1978, Kir'jan and Ševjakova 1984, Swaaij et al. 1985). On the other hand opponents of this idea claim that the greater accumulation of proline following stress is observed in the more sensitive plants (Hanson et al. 1977, Birjukova and Charlamova 1981, Dix and Pearce 1981, Ilahi and Dörffling 1982).

Studies aimed at clarifying the mechanisms of these reactions of plants to the action of abiotic stress agents are usually conducted on plants that are not differentiated in the degree of their sensitivity. On the other hand tests using plant material differing in sensitivity frequently use only one level of the stress agent. It is for this reason that unclear or contradictory conclusions are drawn about the role of proline in the response of plants to the action of stress agents. Thus, in order to determine the effect of low temperature on the content of free proline, we have conducted our experiment on plants we know differ in sensitivity and we have included various intensities of the stress agent.

MATERIALS AND METHODS

For the studies use was made of one-year-old shoots of Norway spruce (*Picea abies* (L.) Karst.) cut from 19-year-old trees growing on an experimental area of the Institute of Dendrology in Kórnik (52°14′46″N and 17°05′00″E), (Giertych 1970). The experiments were carried out on spruces of two Polish populations designated by the numbers: 104 (Wetlina) and 119 (Augustów). Each population was represented by 3 tress treated as replicates. From each tree 3 shoots were cut off and measurements were conducted on them.

Experiments conducted earlier have shown that provenance no. 104 (Wetlina) is characterized by greater sensitivity to the action of low temperatures than provenance no. 119 (Augustów), (Karolewski and Pukacki 1983). This was measured as an increase in electrical admittance of shoots following freezing, which is proportional to the degree of tissue injury (Pukacki 1973, 1978).

The experiments were conducted in mid February, that is during winter rest of the trees. After cutting the shoots they were cooled or frozen in refrigerators in sealed polyethene bags according to the method described earlier (Pukacki 1973, Białobok and Pukacki 1974). The lowering of temperature was controlled, held at a rate of 3°C h^{-1}. The treatment time was 24 h together with the time needed to attain the target temperature of 10, 0, −10, −20, −30 and −40°C depending on the experimental variant. Then the shoots were warmed at a rate of 3°C h^{-1} and placed in a chamber at 0°C. Analyses have been performed on parts of the shoots after 24 h and on others after 108 h (counting from the moment of terminating the chilling/freezing treatment).

One of the analyzed traits was the content of water in the needles subjected to the chilling stress. The content of water in the needles (% of fresh weight) was calculated from the weight difference before and after drying at a temperature of 100°C for 24 h.

The degree of needle injury was evaluated as a measure of changes in the permeability of cytoplasmic membranes. This was determined by the electrolyte leakage method (Dexter et al. 1932), slightly modified as described in Pukacki and Pukacka (1987). Needles were placed in distilled water, obtained using glass distillation apparatus. From each shoot about 1 g of needles was taken and their weight was accurately determined. Then they were placed in test-tubes and distilled water was added 10 cm^3 g^{-1} of needle fresh weight. For the free electrolyte leakage from needles into the water they were subjected to a 20 min. infiltration under vacuum and placed for 24 h at a temperature of 20±2°C. Measurement of electrical conductivity of the solution (a) has been performed with the help of a conductometer OK 102/1 Radelkis and PP 1C type electrodes. The needles were flushed 3 times with distilled water and frozen at a temperature of −60°C for 1 h in order to kill the tissues and cause a complete release of the electrolytes. Next the same quantity of distilled water was added as before (10 cm^3 g^{-1} of needle fresh weight) and after 24 h at a temperature of 20°C the electrical conductivity of the solution was measured again (*b*). A measure of cell injury was defined as the percentage of electrolyte leakage from the tissues i.e. $a(a+b)^{-1}100\%$, where a is the electrical conductivity of solutions from tissues treated with a specific temperature and b the electrical conductivity of solutions from killed tissues.

The level of free proline in needles was analyzed after homogenization in 3% water solution of sulfosalicylic acid with quartz sand added. The concentration of proline was determined spectrophotometrically using the so called fast metod with ninhydrin of Bates et al. (1973). Results have been given in μM of proline g^{-1} dry weight of needles, using the estimates of relations between fresh and dry weight made earlier.

All the results presented in the graphs are averages over three replicates (trees) in each provenance and in each experimental variant.

RESULTS AND DISCUSSION

The results obtained on the content of water in the needles and on the diffusion of electrolytes from them have confirmed earlier information (Karolewski and Pukacki 1983) about the greater sensitivity of provenance no. 104 (Wetlina) compared to provenance no. 119 (Augustów). The calculated critical temperature Tk_{50} (after Pukacki 1973), depending on the experimental variant 24 h and 108 h was respectively −36.2 and −35.4°C for Wetlina and −38.4 and −36.5°C for Augustów. The lowering of the water content in needles also

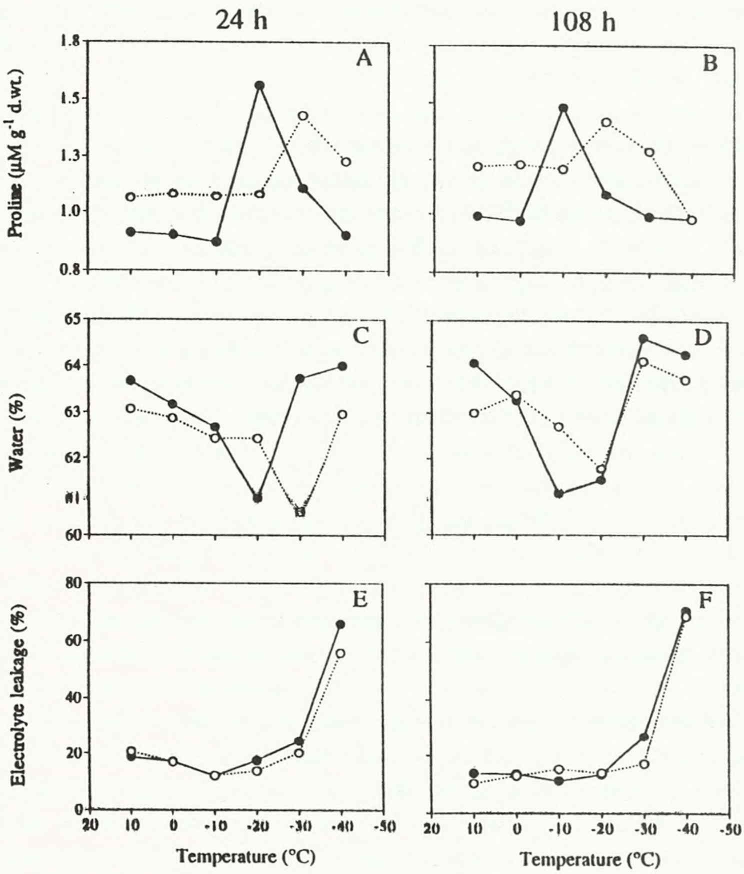

Fig. 1. Effect of low temperature acting for 24 h on the content of free proline (A, B), water (C, D) and electrolyte leakage (E, F) in needles of two Polish populations of Norway spruce (*Picea abies* (L.) Karst.) differing in sensitivity: sensitive Wetlina (‒‒●‒‒) and tolerant Augustów (‒‒○‒‒), measured after 24 h (A, C, E) and 108 h (B, D, F) after termination of exposition to the action of low temperatures for 24 h.

indicates this. It happened at a lesser temperature stress for provenance Wetlina than for provenance Augustów (Fig. 1C and D, Table 1). This was found on the basis of measurements made both after 24 h and after 108 h from the termination of freezing.

Table 1

Significance of differences in the content of free proline, water and electrolyte leakage in needles of Norway spruce between the values obtained for 10°C and the other temperatures used in the study for the two populations: Wetlina (W) and Augustów (A) and between the two populations at each temperature (T). The calculations were made separately for measurements after 24 h and after 108 h following termination of exposition to the action of low temperatures for 24 h.

	Proline						Water						Electrolyte diffusion					
Temp. (°C)	24 h			108 h			24 h			108 h			24 h			108 h		
	W	A	T	W	A	T	W	A	T	W	A	T	W	A	T	W	A	T
10	–	–	**	–	–	***	–	–		–	–	**	–	–		–	–	
0			**			***								*			***	
-10			*	***		**	**			**		*	***	***			***	***
-20	***			**	*	***	***		**	**	**		**				***	
-30	**	***	**			***		***	**		***		**			***	***	***
-40		***	**		*				*		**		***	***		***	***	

* $p<0.1$, ** $p<0.05$, *** $p<0.01$.

Initially (0, −10°C) there were no significant changes in the content of water and of free proline in needles, except for population Wetlina (Table 1). Then (−20°C) the water content in needles also declined (relative to variant 10°C) for provenance Augustów and for measurements after 24 h. Generally the lowering of the level of water was accompanied by an increase in the content of free proline (Fig. 1A and C, B and D, Table 1). The temperature values after which there occurs a rapid accumulation of proline reflect the differences in the degree of sensitivity of the studied populations. The same conclusion was reached by Dreier (1983) for reactions to salt (NaCl). The maximal level of free proline corresponds to the minimal level of water. This regularity occurred irrespective of the time when measurements were made and the population studied (Fig. 1A and C, B and D).

Lack of significant changes in the level of proline may be an indication of the degree of resistance of plants to the given factor. The role of proline in defence and repair mechanisms is documented by results of various studies. First of all it represents a source of energy which is an effective activator of the Krebs cycle and it participates in the rebuilding of chlorophyll (Britikov 1975). Proline is also believed to be a nontoxic intracellular substance protecting against denaturation by osmosis or hydrogen bonding of water (Schobert 1977, Withers and King 1979), and also a compound that protects enzymes and cellular structures from forming Schiff bases (Schobert and Tschesche 1978).

Smirnoff and Cumbes (1989) have shown that proline and its derivatives stabilize enzymes against denaturation by free radicals forming during stress. There are however differences of opinion as regards the magnitude of the role of proline and the intensity of the stress at which it performs a protective function. Our results indicate that more sensitive plants experience stress at relatively small declines in temperature. Thus the accumulation of free proline in more sensitive plants occurred at higher temperatures than in the more tolerant ones (Fig. 1A and B). In this case the accumulation of proline may also be associated with defence mechanisms. It depends on the use of ammonia in the synthesis of proline, the ammonia being a toxic product of the catabolism of amino acids and polyamines.

In needles subjected to temperatures $-30°$ and $-40°C$ substantial lowering of the level of this imino acid was found and it is accompanied by an increase in the content of water (Fig. 1A and B, C and D). This could be caused by the influence of temperature on changes in the water vapor pressure, on the deposition of water in the form of ice on the surface of needles and then absorption of it during defrosting. The increased ability of dead tissues to absorb water is well known (Grzesiuk and Kulka 1981). On the other hand the content of proline at such low temperatures is most probably associated with catabolic processes – deamination of this compound and its effluence to the outside medium (Tesche 1979, Karolewski and Shevyakova 1990).

The dependence of free proline content in needles on temperature is not linear (Fig. 1A, B). This may represent some difficulty in the use of a measurement of the level of free proline as an indicator of low temperature stress experienced by the plant. However field investigations of the effect of frost and drought, such as were performed by Birjukova and Charlamova (1981) point to the possibility of practical use of this method. These authors have found a significant positive correlation between the content of proline in Scots pine needles and the degree of their injury. Such a possibility is also indicated by studies on the influence of other abiotic stress agents, such as toxic gases (Karolewski 1984a, 1989).

Results of the relationships between the content of water and proline in spruce needles and the temperature treatment were similar in character when the determinations were made after 24 h or after 108 h from the termination of exposition of shoots (Fig. 1A and C, B and D). The only difference is that the changes in the level of the studied parameters occurred already at higher temperatures when the measurement was made after 108 h. Probably storage for a period of 108 h of previously frosted shoots has caused increased visible degradation changes already at higher temperatures.

The influence of low temperatures on electrolyte leakage (Fig. 1E, F) did not differentiate the studied populations when the measurement was made after 24 h, but only after 108 h (Table 1). Shape of the curves depended on the length of this period. It is noteworthy that in both the populations a significant and very large change in the electrolyte leakage occurred at a temperature of −40°C (Fig. 1E, F). It occurred only at such temperature at which a lowering of proline occurred (Fig. 1A and E, B and F). Possible within the range of temperatures down to −30°C in spruce needles proline has a stabilizing effect on cytoplasmic membranes. Such a protective function of this imino acid in plants subjected to the action of frost is indicated by Haber et al. (1971). On the other hand, from these and earlier studies (Pukacki 1982) it is known that the critical temperature for Norway spruce needles is within a range of temperatures lower than −30°C.

The result of studies presented in this paper on the content of water, content of free proline and electrolyte leakage from Norway spruce needles indicate that there is a similar mechanism of response to the action of low temperatures in the two studied populations. However this response occurred at a higher temperature in the case of the more frost sensitive population. Our studies indicate that there possibly exists a defence mechanism in plants when the stressing agent is at a tolerable level, however when its action becomes stronger catabolic processes begin to dominate.

The nature of chages in the level of free proline in needles of spruce under the influence of low temperature is very similar to that observed in other species of plants under the influence of other stress agents such as SO_2 and sulphite ions (Karolewski 1984b, Karolewski and Shevyakova 1990), NaCl (Chu et al. 1976, Karolewski 1989b), drought (Ilahi and Dörffling 1982) and cations of toxic metals (Saradhi and Saradhi 1991). In each case, with an increase in the level of the stressing agent we first observe none or only slight increase in the content of free proline then its rapid accumulation and finally after crossing a critical level of tolerance a lowering of the level of this imino acid. This indicates that in the context of changes in the level of free proline we deal with a qualitatively similar reaction of plants to the action of various abiotic stress agents. This confirms the opinion of some investigators who suggest that there occurs an integrated mobilization by plants of physiological and biochemical mechanisms aimed at counteracting the consequences of various types of stress agents (Jones 1978, Chapin 1991).

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Wolna prolina jako wskaźnik wrażliwości świerka pospolitego (*Picea abies* (L.) Karst.) na niską temperaturę

Streszczenie

Określano zawartość wolnej proliny i wody w igłach dwóch polskich populacji świerka pospolitego (*Picea abies* (L.) Karst.), przetrzymywanych przez 24 h w temperaturach: 10, 0, −10, −20, −30 i −40°C. Poziom iminokwasu oznaczano po 24 h i 108 h od ukończenia ekspozycji pędów. Stopień uszkodzenia igieł określano metodą pomiaru wypływu elektrolitu z igieł. Stwierdzono, że poziom wolnej proliny jest czułym wskaźnikiem stopnia wrażliwości badanych populacji świerka na niskie temperatury. Rośliny bardziej wrażliwe reagują już przy mniejszym natężeniu stresu. Charakter zależności zawartości wolnej proliny w igłach świerka pospolitego od niskiej temperatury jest podobny do stwierdzonego we wcześniejszych badaniach z wpływem innych abiotycznych czynników stresowych. Odpowiednio do nasilenia stresu, początkowo brak jest zmian, po przekroczeniu określonego progu występuje gwałtowna akumulacja i ostatecznie spadek poziomu iminokwasu. Analizowana zawartość wody w igłach świerka oraz wypływ z nich jonów dobrze odzwierciedlają stopień uszkodzenia tkanek.