Phenolic compounds level and localization in chilled roots of soybean [*Glycine max* (L.) Merr.]

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The changes in L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity, soluble phenolic compounds and isoflavonoid content in soybean [*Glycine max* (L.) Merr.] roots grown 1-4 days at 5˚C were investigated. Moreover, the localization of intracellular phenolic compounds after 24 h of chilling stress was ultrastructurally determined. The activity of PAL and the levels of soluble phenolic compounds were higher in the chilling-treated roots than in the controls. PAL activity did not correlate with the amount of soluble phenolic compounds in the roots. Enhanced content of isoflavonoids (daidzein, genistein and genistin) in 24 h chilling-stressed roots diminished after 4 days of chilling. At the ultrastructural level, the phenolic compounds in the form of dark deposits varying in shape and size, were visible in the cytoplasm and the vacuoles of both control and chilled roots. A higher frequency of cells with dark deposits in the cytoplasm was noticed in the controls, while vacuoles with tonoplast partly or almost completely covered with these deposits were more characteristic of the cells of chilled roots. In the roots chilling-treated in caffeine presence, deposits disappeared from the cytoplasm, and the vacuoles with clear tonoplast dominated over the vacuoles of the second and third type. The obtained results favour the suggestion that at least part of the dark material in the cytoplasm and in the first type of vacuoles (with clear tonoplast) represented epicatechine-like phenols. The differences between chilled and control roots are discussed.

Key words: chilling stress, *Glycine max,* isoflavonoids, roots, ultrastructural localisation.

INTRODUCTION

Tropical and subtropical plants (e.g. soybean, mungbean, etc.) exhibit marked physiological and biochemical disfunctions when exposed to temperatures below 10˚C. Some effects induced by chilling are mediated by active oxygen species (AOS) (Wingsle *et al*., 1999), because, in spite of their toxicity, AOS also trigger stress tolerance (Dat *et al*., 2000). Plants protect themselves against AOS by activating enzymatic (peroxidases, superoxide dismutases, catalases) and non-enzymatic antioxidants (proline, glutathione, ascorbate, as well as phenolic compounds such as derivatives of hydroxycinnamic acids and flavonoids) (Wingsle *et al*., 1999).

Phenolic compounds derived from *trans*-cinnamic acid are produced by deamination of L-phenylalanine which is catalysed by the enzyme L-phenylalanine ammonia-lyase (PAL, EC 4.1.3.5). Therefore, changes in PAL activity are significant in modulating phenolic compound biosynthesis in plants. Expression of PAL genes is regulated by a diverse array of environmental factors (such as low temperature, UV radiation and pathogens) and is also dependend upon the developmental stage of the plant (Christie *et al*., 1994; Sarma *et al*., 1998). Many studies have focused on the biological activities of

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the phenolic compounds as potent antioxidants (Rice-Evans *et al.*, 1995). The antioxidant activity is mainly due to redox properties, which allow phenolic compounds to operate as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al.*, 1995; Catherine *et al.*, 1996). Phenolic compounds are present in plants as constitutive defensive components, but they can also accumulate in response to various stresses (Dixon & Paiva, 1995; Janas *et al.*, 2002). Moreover, their high content seems to play an essential role in plant protection against low temperatures (Dixon & Paiva, 1995).

Although the subcellular sites of phenolic compound biosynthesis are still a matter of debate they have been observed in the cytoplasm and the nucleus (Wronka *et al.*, 1995), the endoplasmic reticulum (ER) and the cytoplasmic space between cell wall and plasmalemma (Kuraś *et al.*, 1999), but mostly in the vacuoles (Mackenbrok *et al.*, 1992).

The present study was undertaken to determine the effect of chilling stress on PAL activity and the products of the stress (content of phenolic compounds and their intracellular localization).

MATERIALS AND METHODS

Soybean seeds [*Glycine max* (L.) Merr.] var. Aldana obtained from the Institute of Breeding and Acclimatisation of Plants (Radzików, Poland), were used in the experiment. After surface sterilization with a fungicide (Thiuram, Organica-Sarzyna, Poland) seeds were placed in plastic boxes filled with cotton wool wetted with distilled water. When 3-days-old seedlings of uniform length appeared, they were cold-stressed by moving the boxes to a growth chamber at 5°C, in the darkness for 1-4 days. Seedlings growing at 25°C were used as a control.

Extraction and assay of PAL activity in the roots were performed as described by Janas *et al.* (2000). The protein content in the PAL extracts was determined according to the Bradford (1976) method, using bovine serum albumin as a standard.

Soluble phenolic compounds were extracted from the roots with ethanol and were assessed by applying the colorimetric method of Singelton & Rossi (1965). Concentration of phenolic compounds was determined by using chlorogenic acid as a standard.

Isoflavonoids were obtained from a methanol extract of lyophilized roots. The extracts were filtered, reduced in volume by rotary evaporation to the water phase and purified on a C18 column Bakerbond spe Octadecyl (BAKER spe-12G, bed 500 mg). Isoflavonoids were analyzed by High Pressure Liquid Chromatography (HPLC) using a Hewlett-Packard Liquid Chromatograph HP 1100 with a Lichrosorb RP-18 column (4.6 mm × 250 mm) 10 μm C18 reverse phase packing (Altech Associates, Deerfield IL), as described by Graham (1991). The elution protocol was carried out at a flow rate of 1.5 ml min^{-1} using a linear gradient of 5-50% of acetonitrile in water. HPLC grade water was adjusted to pH = 3 with trichloroacetic acid (TCA). Spectrometric detection was at 248 nm. Pure synthetic isoflavonoids (genistein, daidzein and genistin) were used as a standard.

In order to localize the phenolic compounds at the cellular level, 24h-chilled roots as well as control roots were fixed at 0-4°C for 24 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH = 7.2, with 0.1% caffeine addition and subsequently washed in the same buffer (Mueller & Greenwood, 1978). Roots fixed without caffeine were used as an additional control. After postfixation with 2% OsO_4 and dehydration in an ethanol series, root tips were embedded in an Epon-Spurr mixture. Ultrathin sections were cut on Reichert type ultramicrotome and stained with uranyl acetate and lead citrate (Reynolds, 1963). Observations were made on a JEOL 1010 transmission electron microscope at 80 kV.

The number of cells with deposit of electron-dense material as well as deposit intracellular localization were determined on 50 microphotographs from each experimental series, enlarged by × 22000.

Results of PAL activity, soluble phenolic compounds and isoflavonoid levels are the means of 9 replicates in 3 independent experiments. The significance of differences between mean values was determined by the Cochran-Cox test.

RESULTS

Changes of PAL activity in soybean roots are shown in Fig. 1. Whereas in the control roots PAL activity become diminished with time of culture, in the cold treated ones it gradually increased reaching the highest value on 4th day of chilling ($p < 0.05$) (Fig. 1).

In comparison to the roots grown for 4 days at 25°C, a marked increase in the content of soluble phenolic compounds appeared in the 24h-chilled roots ($p < 0.05$), but on the next days of stress, their content subsequently diminished although it was still higher than that in the control material (Fig. 2).

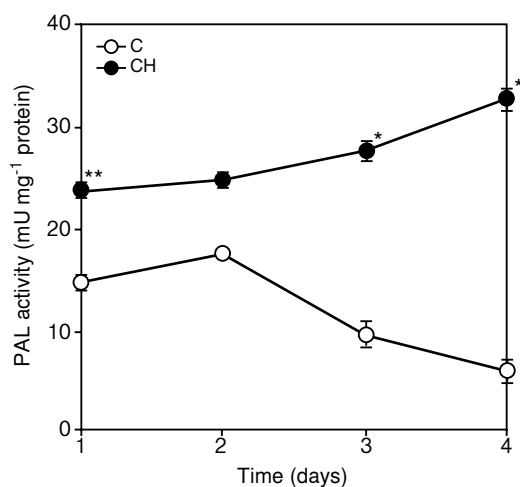Changes in the contents of isoflavonoids in the

FIG. 1. Changes in PAL activity in soybean roots grown at 25˚C (C) and 5˚C (CH), respectively. Data are mean ± SD (n = 9), differences between initial and final value are significant at $p < 0.005$ (*) and $p < 0.025$ (**), respectively.
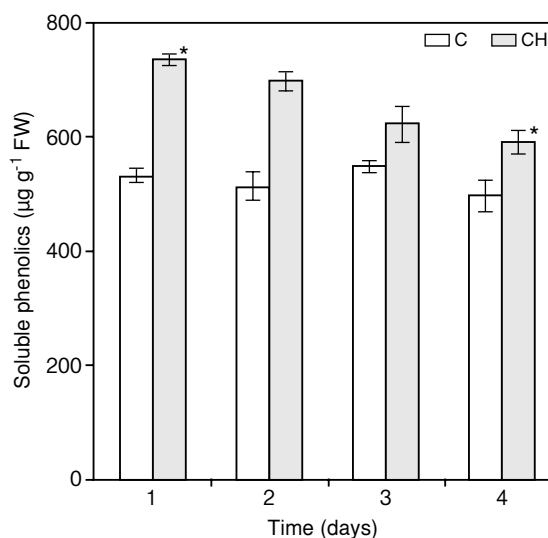
FIG. 2. Changes in soluble phenolic compounds in soybean roots grown at 25˚C (C) and 5˚C (CH), respectively. Data are mean ± SD (n = 9), differences between initial and final value are significant at $p < 0.005$ (*).

control and chill-treated roots are shown in Fig. 3. Their content rose in the roots 24 h after transfer of the seedlings from 25˚C to 5˚C and then dropped during the following days of chilling. The levels of daidzein, genistein and genistin increased by about 100% ($p < 0.025$), 60% ($p < 0.025$), 35% ($p < 0.05$), respectively in the roots after 24 h of low temperature treatment in comparison to the control (Fig. 3).

Ultrastructural investigation revealed the presence of phenolic compounds in the cytoplasm and vacuoles of the control and cold-treated root meristem of the cells (fixed with and without caffeine)

(Figs 4-11). Sporadically, these compounds were observed also within the cell walls in the vicinity of intercellular spaces (Fig. 11).

In the cytoplasm, numerous deposits of electron-dense material regular in shape and ranging in size from 0.01 to 0.02 μm, were randomly dispersed in the vicinity of vacuoles and cell walls (Figs 4-5). Occasionally, aggregates of these deposits were observed in the cytoplasm near the tonoplast (Fig. 5).

In the vacuoles, two types of deposits were identified. The deposite of the first type were single and large (0.3 to 2.3 μm), globular or lens-shaped (Fig.
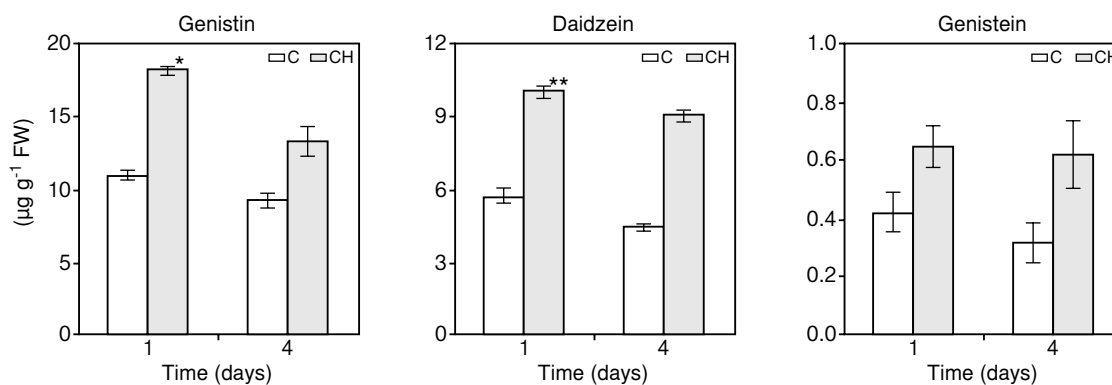

FIG. 3. Changes in isoflavonoid contents of soybean roots grown at 25˚C (C) and 5˚C (CH), respectively. Data are mean ± SD (n = 9), differences between initial and final value are significant at $p < 0.005$ (*) and $p < 0.025$ (**), respectively.
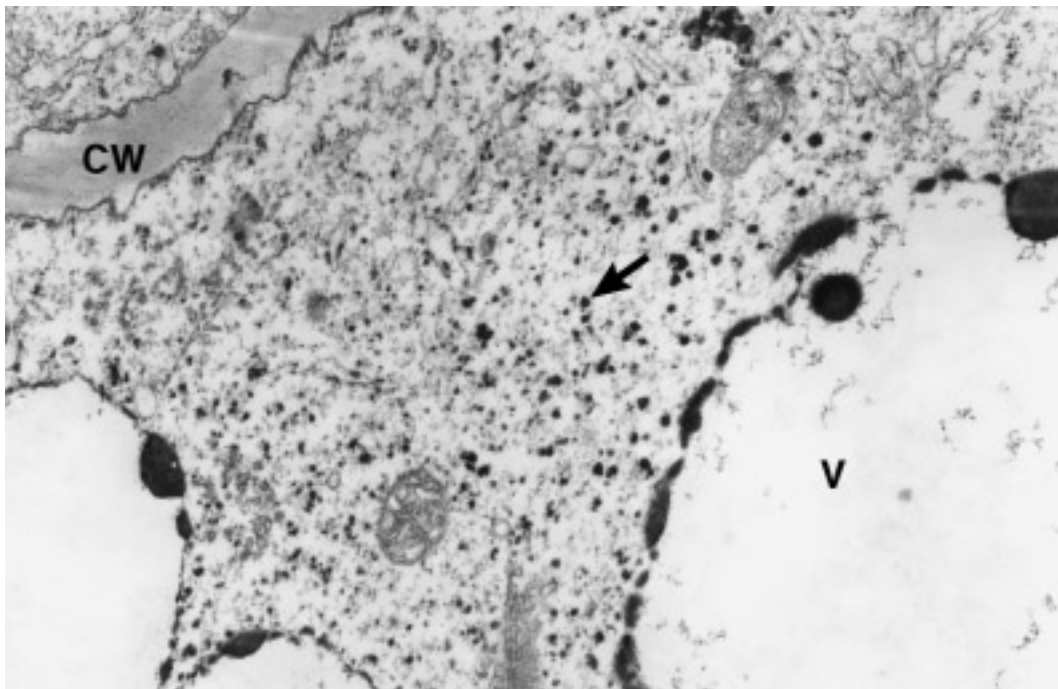
FIG. 4. Numerous small deposits of electron-dense material (arrow) dispersed in the cytoplasm of control root meristem cells fixed with caffeine (×22000).
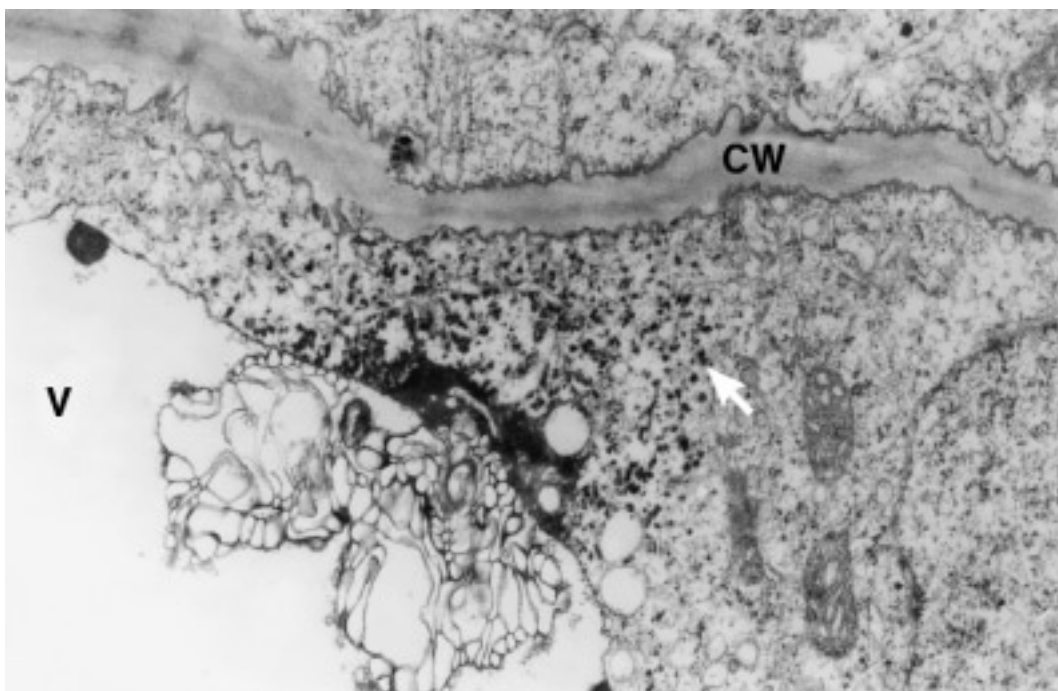

FIG. 5. Aggregates of small dark deposits in the vicinity of vacuoles (×22000).
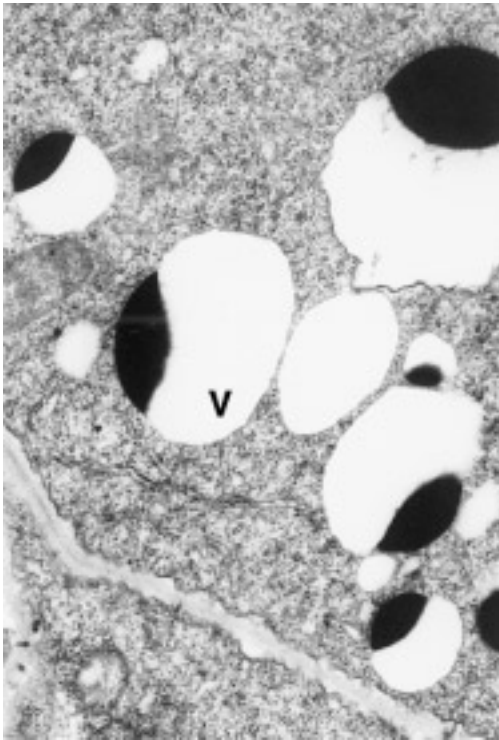
FIG. 6. Vacuoles with clear tonoplast and large single deposits of electron-dense material (first type vacuole); control material fixed without caffeine (×22000).
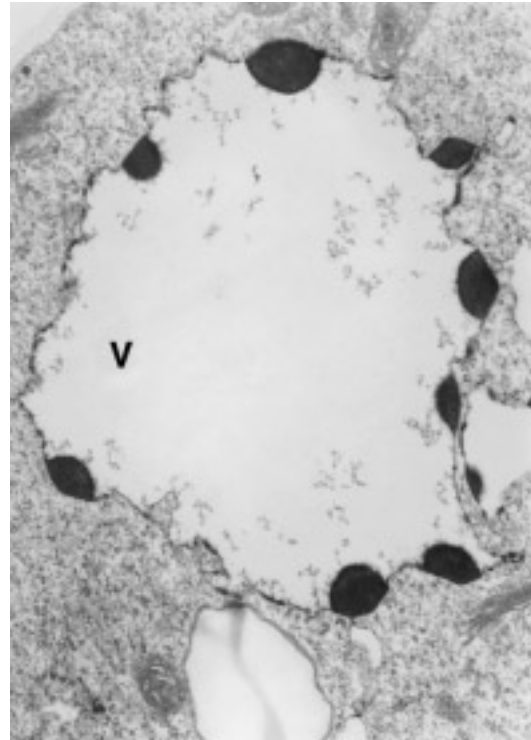

FIG. 7. Small deposits partly covering tonoplast (second type vacuole); cold-treated *Glycine max* root meristems fixed without caffeine (×17600).
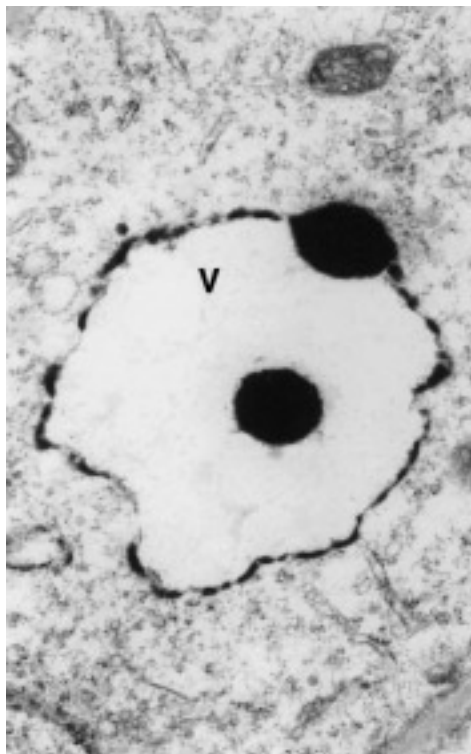
FIG. 8. Numerous deposits almost completely covering the tonoplast (third type vacuole); control material fixed with caffeine (×22000).

FIG. 9. Large dark deposits with various size partly covering the tonoplast; control material fixed with caffeine (×17600).

FIG. 10. Deformed vacuoles with numerous deposits of electron-dense material in cold-treated root meristematic cells fixed with caffeine (×22000).

FIG. 11. Deposits of electron–dense material within the cell wall facing an intercellular space; control roots fixed with caffeine (×22000).

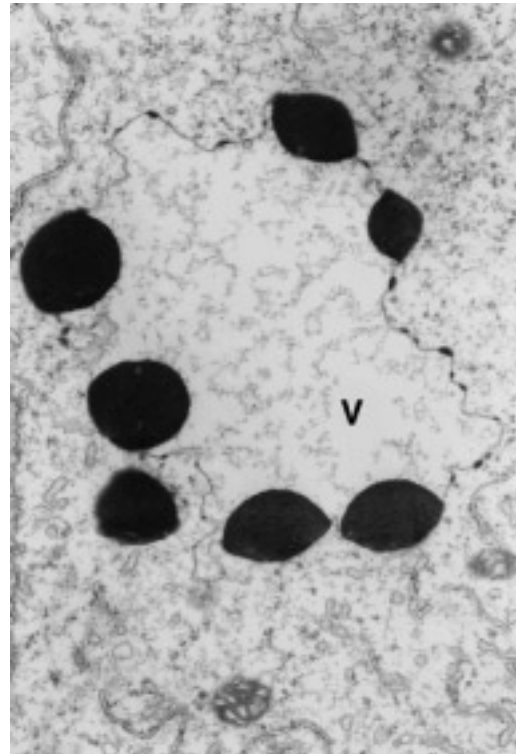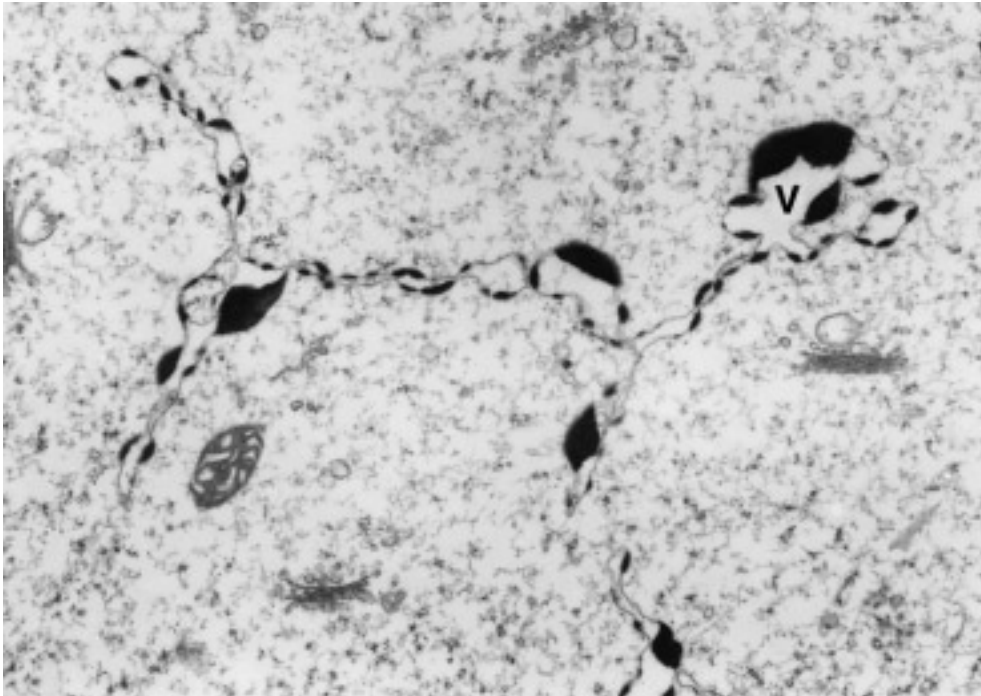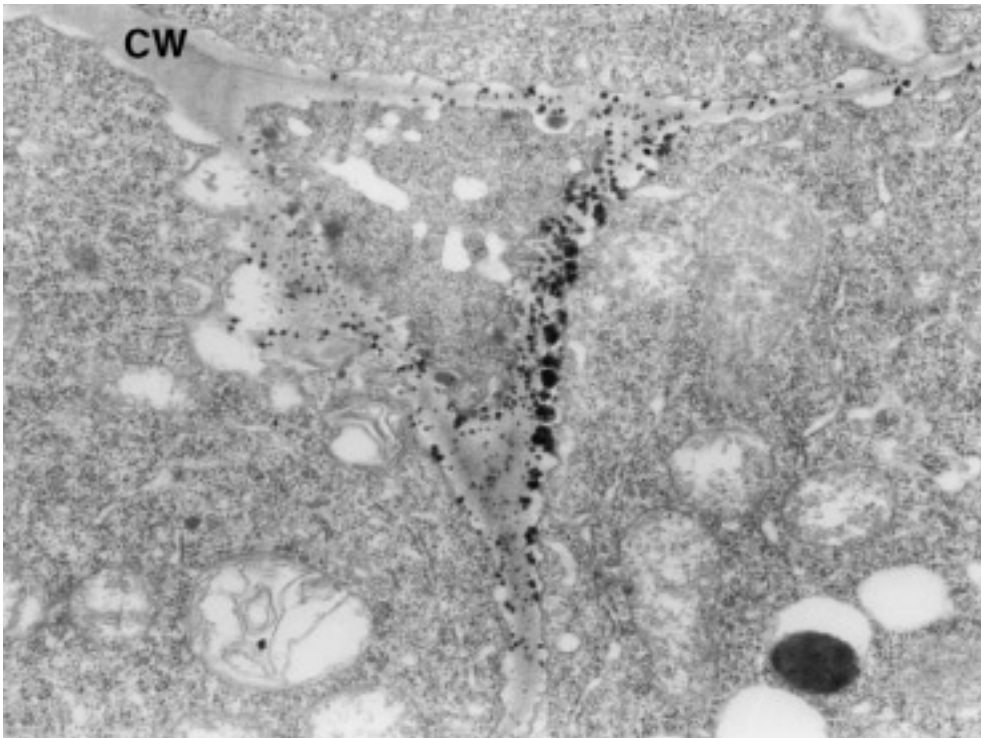TABLE 1. Mean number of root meristematic cells (per section) having deposits of electron-dense material in their cytoplasm and frequency of vacuoles (types) per meristematic cell containing the same material

Temp. (°C)	Caffeine	Cytoplasm	Vacuoles		
			Type a[1]	Type b[2]	Type c[3]
25	–	7.29	74.31	20.62	5.05
	+	18.18	52.19	35.68	10.60
5	–	3.14	54.95	32.36	13.35
	+	0.00	74.70	22.76	2.78

[1] one-two large deposits and clean tonoplast
[2] small deposits partly covering tonoplast
[3] small numerous deposits almost completely covering tonoplast

9), mostly localized adjacent to the tonoplast. The deposites of the second type were much smaller (0.04 -0.09 μm) numerous, usually lamellar in shape, covering the tonoplast partly or almost completely (Figs 7-10). Under this consideration, three types of vacuoles were distinguished: vacuoles with clear tonoplast and one or two large deposits of electron-dense material (Fig. 6) and vacuoles with tonoplast partly (Fig. 7) or almost completely (Fig. 8) covered with numerous deposits, small and irregular in shape. Some vacuoles of the second and third type were significantly deformed; however their number was higher in the chilled roots (57%) than in the controls (23%) (Fig. 10).

In spite of the localization of the phenolic compounds some differences between the chilled and control material appeared. In the chilled roots fixed without caffeine, the number of cells with the above-mentioned deposits in the cytoplasm was diminished, while in the roots fixed with caffeine, the cells with such deposits disappeared (Table 1). On the other hand, the presence of caffeine in the control roots doubled the number of cells containing phenolic deposits in the cytoplasm (Table 1).

DISCUSSION

Temperature is an important environmental factor affecting phenylpropanoid synthesis in various plants (Dixon & Paiva, 1995). In the present study, it was shown that the activity of PAL (the key enzyme of phenylpropanoid synthesis), as well as the levels of the soluble phenolic compounds increased in chilling-treated roots of soybean. In contrast, in the roots of seedlings grown in the darkness at 25°C, the activity of PAL decreased. Decreased PAL activity as

well as low levels of soluble phenolic compounds at optimum temperature and darkness, are well-established observations (Zucker, 1965). Enhanced PAL activity and accumulation of phenolic compounds in response to chilling were demonstrated in various plants (Christie *et al*., 1994; Solecka, 1997; Solecka *et al*., 1999; Cantwell *et al*., 2002). PAL is turned over rapidly at room normal temperature (Lawton *et al*., 1980), but the mechanisms of PAL activation in the cold are less known.

HPLC analyses of roots revealed increased isoflavonoid contents (both aglycones-daidzein and genistein, and glycosyl conjugate of genistein-genistin) after 24 h of chilling and subsequent decreased contents during prolonged chilling. These results are analogous to those presented earlier in soybean roots (Zhang & Smith, 1996).

The results presented in this paper suggest that the effects of chilling on PAL activity do not coincide with those on phenolic compound accumulation in the roots. Thus, it is possible that these compounds may originate from a pre-existing form, e.g. a cellular structure or conjugates which act as a reservoir of aglycones and can be mobilized if needed (Courtois & Guern, 1980; Janas *et al*., 2002; Piślewska *et al*., 2002).

Cytological studies on normal and stressed plants have indicated the intracellular sites of synthesis and accumulation of phenolic compounds. As it has been shown by Schutze & Rudeff in 1865 (in Hayat, 2000), phenolic compounds can be visualised within the cells due to their reaction with OsO_4. Therefore, deposits of electron dense material in the root meristematic cells of *Glycine max* var. Aldana most probably represent phenolic compounds.

Phenolic compounds are considered to participate in plant defence against biotic and abiotic stresses; they were found in i) *Lactuca* sp. in great amounts after *Bremia lactucae* infection (Sedlarowa & Lebeda, 2001), ii) cold-treated leaves of *Brassica napus* (Stefanowska *et al.*, 2002) and iii) in the chilling roots of *Pachyrhizus erosus* (Cantwell *et al.*, 2002). In *Glycine max* var. Aldana they were observed not only in chilled roots but also in those growing at 25˚C. This may suggest that the increase of phenolic compounds in the chilled roots is primarily due to the increased levels of precursor compounds (Janas *et al.*, 2002). Similarly, phenolic compounds were described in non-chilled root cells of *B. napus* (Wronka *et al.*, 1995); according to their opinion, phenolic compounds appeared due to exposure to harsh conditions of soil. It is possible that in *Glycine max* var. Aldana, a plant originating from a hot oriental climate, phenolic compounds might have appeared in the root cells as constitutive compounds during the subsequent stages of soybean acclimation to the lower northern European temperatures. Their presence in the roots of the non-cold stressed and more sensitive *Glycine max* var. Essor (Mikiciński, personal communication) might additionally support the above suggestion.

In the cells of both control and chilled roots of soybean, as in other plants (Wronka *et al.*, 1995; Kuraś *et al.*, 1999; Sedlarowa & Lebeda, 2001; Stefanowska *et al.*, 2002), phenolic compounds in the form of electron-dense deposits were distributed mostly in the vacuoles, sometimes in the cytoplasm, and sporadically in the cell wall or its vicinity. The appearance of electron-dense deposits in the cell wall is not peculiar, because the amount of phenolic acids released after alkaline treatment of cell wall material decreased in 24 h chilling-treated roots of soybean seedlings (Janas *et al.*, 2000). A dramatic modification of hemicelluloses during the first hours of chilling was observed in winter wheat roots (Zabotin *et al.*, 1998). It seems that chilling results in a decrease of cell wall polysaccharides, and phenolic compounds such as phenolic acids and flavonoids cannot form links with them, so they remain in the soluble pool of phenolics. Contrary to *B. napus*, phenolic compounds in *Glycine max* were never observed within the nucleus (Wronka *et al.*, 1995; Kuraś *et al.*, 1999). On the other hand, in the leaves of *B. napus* after cold-treatment, phenolic compounds were never observed in the vacuoles, probably due to their leaking to the cytoplasm during the fixation

procedure (Stefanowska *et al.*, 2002).

So far, the chemical character of phenolic compounds visualized with OsO_4 (Transmission Electron Microscopy, TEM) remains unknown. Solecka *et al.* (1999) and Janas *et al.* (2000) using HPLC analyses have identified phenolic compounds in cold acclimated leaves of *B. napus* and soybean roots as derivatives of hydroxycinnamic acids, mostly in esterified form. In the present paper we demonstrated an increase in glycosyl conjugate of genistein-genistin in chilling-treated roots. Estrification and glycosilation of phenolic compounds such as flavonoids, and derivatives of hydroxycinnamic acids, might allow their transportation to the vacuoles (Dixon & Paiva, 1995) where along with peroxidase and ascorbic acid can function as a mechanism for AOS (e.g. H_2O_2) scavenging in plants (Takahama & Oniki, 2000). The antioxidant properties of phenolic compounds (e.g. flavonoids and hydroxycinnamic acids) are well-documented (Chu *et al.*, 2000; Heim *et al.*, 2002).

In addition, caffeine added to the fixative not only prevented leaking of phenols from the vacuoles to cytoplasm (Mueller & Greenwood, 1978) but also allowed visualization of epicatechin-like phenolic compounds (Mueller & Beckman, 1974; Mueller & Greenwood, 1978). Therefore, adding of caffeine to the fixative was expected to contribute to distinguishing of this particular group of phenolic compounds in the soybean root cells.

Contrary to our expectations, caffeine allowed to observe changes in the number of cells having phenolic compounds in their cytoplasm and also to distinguish specific types of vacuoles, as when compared with the roots, fixed without this alkaloid. In the control roots, an increase in cell frequency of the cells with phenolic deposits in the cytoplasm and the vacuoles with tonoplast partly or almost completely covered with deposits was observed. In the chilled roots fixed with caffeine, these deposits disappeared from the cytoplasm but they remained in the first type of vacuoles, i.e. with clear tonoplast and 1-2 large deposits. The observed accumulation of large phenolic deposits in the vacuoles of chilling-treated roots is in accordance with our earlier suggestion about the role of these substances as antioxidants, which together with peroxidase function as an H_2O_2-scavenging mechanism (Takahama & Oniki, 2000). Taking into consideration that caffeine stabilizes phenolic compounds within the vacuoles (Mueller & Greenwood, 1978) as well as it allows detecting of epicatechin-like phenolic compounds (Mueller &

Beckman, 1974; Mueller & Greenwood, 1978), it cannot be excluded that some large deposits in the vacuoles consist of this material. If the above suggestion is correct, the presence of the first type of vacuoles in a smaller number and of the other two types in a greater number, as well as the higher frequency of cells with phenolic deposits in the cytoplasm of the control roots after caffeine addition, remain obscure. Changes in the chemical character of the phenolic compounds after cold stress can be the only possible explanation of these observations.

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