EXPRESSION OF PHENYLAMIDES IN ABIOTIC STRESS CONDITIONS

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Summary. The formation of phenylamides – conjugates of aliphatic di- and polyamines and hydroxy cinnamic acids – was investigated in two plant - abiotic stress model systems: tobacco-water excess stress and bean-high temperature stress. The concentration of free radicals was also determined. It was established that in both systems the stressed leaves synthesized a set of plant specific phenylamides lacking in the controls. Hence, having also in mind that phenylamides are scavengers of toxic free radicals, it may be assumed that they are components of a general defense system in plants against stresses of different origin.

Key words: phenylamides, polyamines, free radicals, tobacco, bean, abiotic stress

Abbreviations: PhA – phenylamides; EPR – electron paramagnetic resonance; UV – ultra violet; TLC – thin layer chromatography; PFD – photon flux density

Introduction

Phenylamides are constituents specific to plants, conjugates of hydroxycinnamic acids and amines. In the present paper conjugates of aliphatic di- and polyamines** (not aromatic amines) are to examined. Polyamines form covalent amidic bond with one

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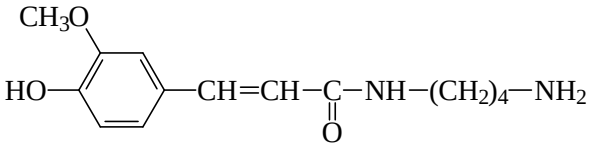
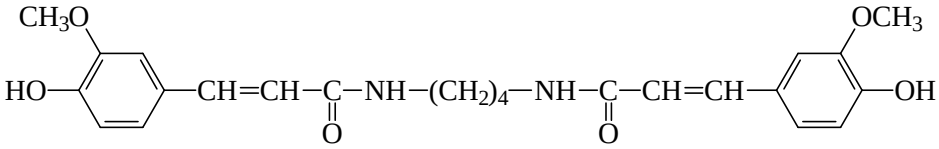
** For the sake of brevity, only “polyamines” - as a common term - will be used further

molecule of hydroxycinnamic acids giving rise to basic PhA, or with two molecules of these acids, resulting in neutral PhA (Fig. 1).



Ferulylputrescine



Diferulylputrescine

Fig.1. An example of phenylamide compounds: ferulylputrescine, a basic conjugate of one molecule ferulic acid with one amino group of putrescine; diferulylputrescine, a neutral conjugate of two ferulic acid molecules with both amino groups of putrescine

PhA are endowed with both polyamine and hydroxycinnamic acid characters, and hence have dual functions. They can combine growth and development regulatory functions of polyamines (Martin-Tanguy, 1985) and toxic free radical scavenging, antioxidative and wall strengthening properties conveyed by hydroxycinnamic acids (Clarke, 1982; Fry, 1986; Bors et al., 1989; Ohnishi et al., 1994; Volpert et al., 1995).

Data about their intracellular, apoplastic and cell wall localisation (Clarke, 1982; Vallee et al., 1983; Langebartels et al., 1991), as well as about their involvement in germination, organogenesis (rooting, tuberisation), induction of flowering, pathogenesis and disease resistance are reported (Martin-Tanguy, 1985; Flores and Martin-Tanguy, 1991). However, the information about PhA expression in abiotic stress situations is very scarce. To our knowledge, it is limited to the formation of PhA – as a stress response – in leaves of tobacco fumigated with ozone (Langebartels et al., 1991), and suffering K^+, Ca^{2+}, Mg^{2+} and P deficiency (Deletang, 1974), as well as in S-starved tobacco cell suspension cultures (Klapheck, 1983).

For this reason the aim of our work was to investigate PhA expression in other plant-abiotic stress systems, i.e. to establish whether PhA involvement in abiotic stresses is an incidental or a more general phenomenon. Taking into account that PhA are scavengers of free radicals (Bors et al., 1989), the concentration of the latters was determined.

Two plant-abiotic stress model systems were used: tobacco-water excess stress and bean-high temperature stress.

Materials and Methods

Plant material and growth conditions

Tobacco. Plants of *Nicotiana tabacum* L. (oriental cv. Nevrokop 5) were used. They were grown in sterilized soil under greenhouse conditions (20–25 °C average day temperature, 60–80 % relative humidity, and 240 mol.m^{-2}.s^{-1} PFD). Uniformly developed plants of about three months were used in the experiments.

Bean. Plants of *Phaseolus vulgaris* L. (cv. Cheren starozagorski) were used. They were grown as a water culture in a growth chamber (23–25 °C, 60–80 % relative humidity, and 120 mol.m^{-2}.s^{-1} PFD, at a photoperiod 12 : 12/day : night). Plants of 12–14 days were used in the experiments.

Stress treatments

Tobacco - water excess. Water excess induces in oriental tobacco dot-like necrotic spotting on leaves; this is the physiological disorder named “sharilka”. The water excess treatment of plants was carried out according to a procedure described earlier (Edreva et al., 1972).

Leaves for analysis were taken from the middle stalk position of about 20 plants. Damaged areas of leaves were selected and discs of 1.0 cm in diameter were cut, containing an equal number of necrotic spots. Middle leaves from not-treated plants served as controls.

Bean - high temperature stress. Plants were subjected to a high temperature treatment (52°C) for 5 h (Yordanov, 1981) resulting in wilting, or in same cases injury of leaves. For analysis an average sample from primary leaves of about 25–30 plants were taken. Leaves of not-treated plants served as controls.

Methods

1. Phenylamides

The procedure of Deletang (1974) using fresh leaf material was applied.

1.1. Quantitative determination.

PhA were determined by a combination of ion exchange column chromatography, thin layer chromatography and spectrophotometry. The content of PhA was calculated as a difference of total and free polyamine contents.

1.2. Qualitative analysis

1.2.1. Chromatography. Extracts containing PhA were separated by bidimensional ascending thin layer chromatography on cellulose plates, 0.1 mm (Merck) in the system *n*-butanol : ethanol : water = 4 : 1 : 2 (I direction) and acetic acid : water = 15 : 85 (II direction) (Edreva and Hadjiiska, 1980). The PhA were located on the chromatograms under UV light as blue fluorescing spots; fluorescence is due to the hydroxy cinnamic acid moiety of PhA. Then the chromatograms were sprayed with 1 % solution of ninhydrine in pyridine : acetone = 5 : 95, resulting in violet coloring of spots; it is due to the polyamine components of PhA.

1.2.2. Alcaline hydrolysis (release of the hydroxycinnamic acid components of PhA). Fluorescing spots of PhA were eluted from the chromatograms with 70 % methanol. Eluates were hydrolyzed with 2 N NaOH at 110 °C for 4 h, neutralized and developed using bidimensional ascending TLC (Edreva and Hadjiiska, 1980). The resulting hydroxycinnamic acids were identified by the UV spectra of their eluates and by reference substances.

1.2.3. Acid hydrolysis (release of polyamine components of PhA). Eluates of PhA were hydrolyzed with 6 N HCl at 90°C for 24 h, neutralized and separated using one dimensional ascending TLC on cellulose plates, 0.1mm (Merck) (Hammond and Herbst, 1968). The resulting polyamines were developed with ninhydrine and identified by reference substances.

2. Free radicals

The concentration of free (unpaired) electrons corresponding to the concentration of free radicals was determined. The method of the electron paramagnetic resonance was used (Alger, 1968). The measurments were done on lyophilized leaf material, using a EPR spectrometer ER 200 D-SRC, Brucker. A 100 kH modulation of magnetic field in X diapason was applied.

Results

Tobacco

In the leaves of not-treated (control) tobacco plants no PhA were found which confirms the data of Deletang (1974) and Martin-Tanguy (1985). In the stressed leaves 2 components having blue fluorescence in UV light and reacting positively with ninhydrine were established (Fig. 2). Acid hydrolisis of eluates of these components showed the presence of the diamine putrescine and the triamine spermidine; alcaline

hydrolysis proved the presence of hydroxycinnamic acids, namely caffeic and ferulic acids (Table 1). These results proved the PhA character of the above two components induced by water excess stress. The content of conjugated (as PhA) polyamines is given in Table 2.

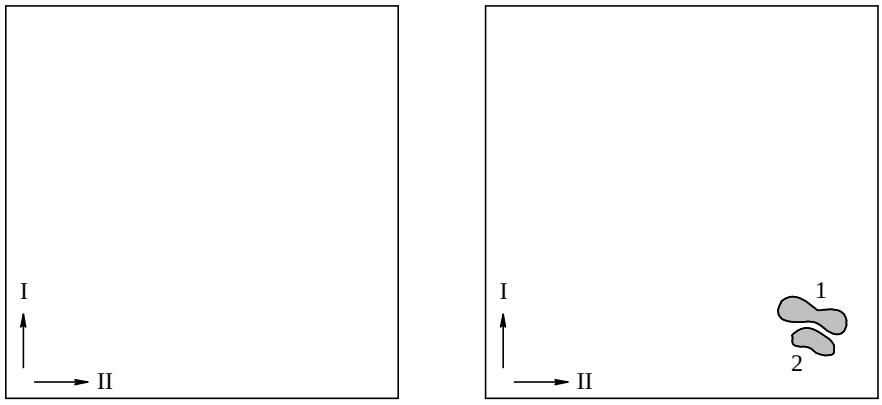


Fig.2. Thin layer chromatogram of phenylamides in leaves of tobacco plants subjected to a water excess stress. Control - leaves of not treated plants. Two spots (No1 and No2) having phenylamide characteristics - blue fluorescence in UV and positive reaction with ninhydrine (violet color) are present in the stressed leaves. No such compounds are observed in the control. Two dimensional ascending thin layer chromatography. I direction – *n*-butanol:ethanol:water = 4:1:2; II direction – acetic acid:water = 15:85

An increased concentration of free radicals was recorded in the stressed leaves (Table 3).

Table 1. Components of phenylamides in leaves of tobacco and bean plants subjected to water excess stress and high temperature stress, respectively. The same components are observed in the leaves of control bean plants. Polyamine components are released by acid hydrolysis, and hydroxy cinnamic acid components – by alcaline hydrolysis of eluates of spots No 1 and No 2 in tobacco and of spots A, B, C and No 1–No 9 in bean

Plants	Polyamines	Hydroxy cinnamic acids
Tobacco	Putrescine Spermidine	Caffeic Ferulic
Bean	Putrescine Spermidine Cadaverine	Ferulic p-Coumaric Caffeic

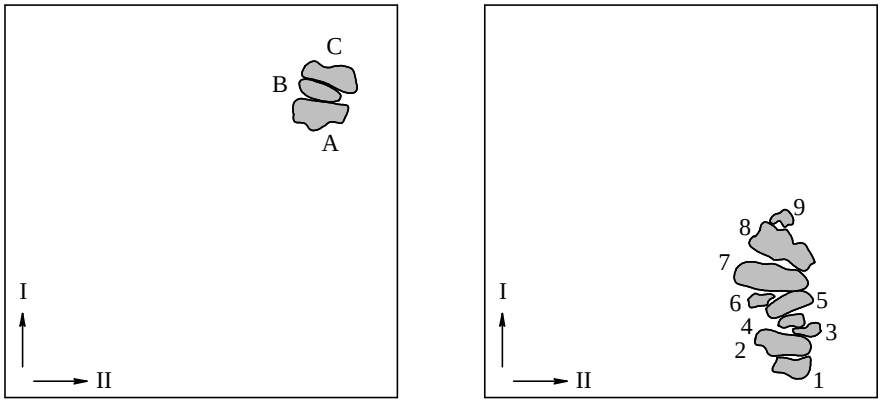


Fig. 3. Thin layer chromatogram of phenylamides in leaves of bean plants subjected to a high temperature stress. Control - leaves of not treated plants. Nine spots (No 1–No 9) are present in the stressed leaves, and three spots (A, B, C) in the control; they have the same phenylamide characteristics as the spots in Fig.2. Thin layer chromatography: explanations as in Fig.2

Table 2. Content of conjugated aliphatic polyamines (nM/g f.w. and % of controls) in leaves of tobacco and bean plants subjected to water excess stress and high temperature stress, respectively. Controls - leaves of not treated plants. The polyamines are conjugated with hydroxy cinnamic acids

Plants	Tobacco		Bean		
Variants	Control	Water excess stress	Control	High temperature stress	
Polyamines	nM/g f.w.		nM/g f.w.		% of controls
Spermidine	0	42	171	431	252
Putrescine	0	98	193	611	316
Cadaverine	-	-	140	581	415

Bean

In leaves of the control bean plants 3 blue fluorescing in UV light and ninhydrine positive spots (A, B, C) were detected. The high temperature stress brought about the appearance of 9 components (No 1 - No 9) having the above characteristic but with quite different chromatographic behaviour as compared to the controls (Fig. 3). Acid hydrolisis of eluates of these components in both the control and stressed leaves showed the presence of the diamines cadaverine and putrescine and of the triamine

Table 3. Concentration of free (unpaired) electrons (corresponding to free radical concentration) (spin/g) in leaves of tobacco and bean plants subjected to water excess and high temperature stress, respectively. Controls – leaves of not treated plants

Tobacco		Bean	
Control	Water excess stress	Control	High temperature stress
3.8×10^{15}	4.6×10^{15}	9.3×10^{15}	9.6×10^{15}

spermidine. Alcaline hydrolisis pointed to the presence of hydroxy cinnamic acids (ferulic, caffeic and p-coumaric) (Table 1). All these data proved the PhA nature of the above compounds. It must be noted that no information about occurrence of PhA in bean is available. The content of conjugated (as PhA) polyamines is presented in Table 2; it is highly increased in the stressed leaves in comparison with the controls.

The free radical concentration of bean leaves subjected to a high temperature treatment is slightly increased (Table 3).

Discussion

Our studies have shown that in bean plants dramatic qualitative and quantitative shifts in PhA pattern following high temperature stress take place. It is also demonstrated that in tobacco leaves PhA are abnormal constituents appearing as a response to water excess stress. Taking also into account the data of Deletang (1974), Klapheck (1983) and Langebartels et al. (1991) concerning other tobacco - abiotic stress systems, it can be concluded that the involvement of PhA in abiotic stress situations is not an incidental phenomenon in plants. Moreover, the documented involvement of PhA in pathogenesis and disease resistance (Martin-Tanguy, 1985) points to their general importance in stresses.

A protective role of PhA can be admitted. This assumption is based on the information that PhA act as scavengers of free radicals toxic to the cell (Bors et al., 1989); moreover, cell wall strengthening properties of PhA are suggested (Clarke, 1982) due most probably to the hydroxy cinnamoyl moiety (Fry, 1986); an antioxidative character could be conveyed by both polyamine (Tadolini, 1988) and hydroxy cinnamic acid (Ohnishi et al., 1994; Volpert et al., 1995) components of PhA. It can be supposed that by conjugation of hydroxy cinnamic acids with polyamines the cytotoxicity of the free forms of these acids is regulated, and their antioxidative and antiradical effect is coupled with the antioxidative properties of polyamines.

In the systems studied, the assumption of protective action of PhA is substantiated by the fact that in bean leaves the free radical level is only slightly increased

and damage is not too severe (wilting), whereas in tobacco the free radical concentration is increased to a higher extent and damage is severely expressed (necrosis). Correspondingly, the PhA variability and content in bean is higher than in tobacco.

Conclusion

Expression of PhA in two plant - abiotic stress systems is established. Taking also into account the involvement of PhA in other abiotic stresses as well as in pathogenesis, they can be considered as components of a general defense system in plants against stresses of different origin. Most probably, the protective effect of PhA is mainly due to their free radical scavenging properties.

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