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TRANSLOCATION OF EXOGEN PHENOLOIDS AND ALKALOIDS IN ACCEPTOR PLANT – HISTO- AND PHYTOCHEMICAL CHARACTERISTICS

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Phenoloids with allelopathic effect (juglone, catechin, tannin, gallic acid, t-cinnamic acid, caffeic acid, coumarin, thymol, salicin in 1 mM concentration) cannot be detected after the absorption from the acceptor plant (bean) by the applied selective analytical method (TLC densitometry). Their localisation can be determined by histochemical reagents (ferrichloride, potassium bichromate, sodium hydroxide). In the foliage leaves of intact and excised bean plants they are present mainly in the parenchymatous elements of the vascular tissue already on the 3rd day, at the beginning of wilting.

Some substances (tropanes) out of the studied allelopathic alkaloids (atropine, scopolamine, belladonnin, tropine and caffeine) can be detected only in small amounts (7–8%) or only in traces in the leaf. Others (e.g. caffeine) accumulate in substantial amount (almost 200%) in their original form. Alkaloids, as well as phenoloids, can be detected in leaf tissue by histochemical methods (Dragendorff and Mayer reagents).

Key words: alkaloids, allelopathy, densitometry, histochemical reactions, juglone, phenoloids

INTRODUCTION

Interactions between plants take place partly by the mediation of their chemical substances. This type of interaction is important both from theoretical and practical viewpoint, especially today, when obtaining sufficient knowledge about substances of natural origin and possibly their application is a key issue in nature conservation and nature protective agriculture (Freedman 1989). Recently, the term allelochemistry, in a wider sense ecological biochemistry, including interactions with animals, has been used in ecological literature (Harborne 1989, Schlee 1986), which refers to the fact that substances dissolving out of plants can be identified by phytochemical methods (or the composition of the exudates can be identified). Furthermore, their chemical construction is known, the plant physiological or even biochemical effect of a substance or substance group can be determined precisely.

Although there is a great number of observations and an extensive literature in connection with the ecological-biological (including allelopathic) effect of special (secondary) plant metabolites (Rice 1984), the biological (plant physiological, histochemical) interpretation of the effects has not been carried out so far. The main reason for this is the fact that biological interactions are the summations of multifactorial effects (Paul and Clark 1989). The complicated and very complex phenomena can only be interpreted if the transformation of the special plant metabolites is studied with model experiments. When tracing the in vitro changing of these metabolites it is supposed to be important to decide first if an inhibitory (or perhaps stimulating) effect originates at all in the course of a biotest. Our goals could not include studying the metabolic pathway of transformed (or not transformed, i.e. stable) metabolites in the tests. From the most important metabolite groups, which also include the highest number of substances, phenoloids and alkaloids were studied by the chosen biotests, with intact and excised horse-bean and bean plants.

To achieve this it was necessary to apply time-saving separation techniques, which ensure the selective separation and detection of the applied substance possible out of the test plants. The methodological tasks included the adoption of published phytochemical methods (Wagner et al. 1983, Tyihák 1979, Sherma and Fried 1991, Jork et al. 1989), modification of extraction process, separation and detection of demanded substances from the extract by thin layer chromatography following densitometry.

The traditional light microscopic method was applied for the histochemical studies.

Internationally, Hashimoto's (1989) histochemical chromatographic (GC, HPLC, MS) and microchemical results are worth to emphasize, although his studies carried out with an automatic micromanipulator concern endogenous substances (crystals, oil droplets, concentrated substance of reaction products).

METHODS

Morphological characteristics

To verify the effect of cytokinin Pozsár (1978) used decapitation and imbibition with excised plants successfully, since the selective effect comes across better by using this. This method was applied in testing allelo-

chemical substances, too. The germinating seeds of bean cv. "Inka" with a suitable sensitivity were placed 1 cm deep in 20 cm × 30 cm boxes filled up to 10 cm with river-sand when the radicle appeared. Plants were watered by Knop-type nutritive solution in equal amounts and at even time periods. When the primary foliage leaves have fully developed (the initiative of the first pinnate trifoliate leaf was discernible), decapitation was started. This stage was ensured for 10 days (decapitation being carried out continuously). Following this all the plants were cut at 2 cm above ground level (root was removed) and the remaining, but already yellowish cotyledons were carefully removed. Plants with the same developmental stage and height, treated as above, having dark green primary foliage leaves were placed in the solution of allelochemicals. Ten plants were placed in each Erlenmeyer flask filled up to 5 cm with the solutions, with 3 parallels in each treatment (altogether 30 plants per treatment). Distilled water was used as control. Solutions were also prepared with distilled water. Evaporation of water and aqueous solutions could be diminished by wrapping the upper part of the flask, excluding the plants, in aluminium foil.

During observations further treatments (decapitation) were not carried out, consequently some of the control plants developed flowers or even pods.

The chemical reaction of the substances and the solutions containing allelopathic substances was measured in each case. 1000 and 100 ppm concentrations were applied from the following: hydrochloric acid, sulphuric acid, citric acid, indol acetic acid, juglone, atropine sulphate, thymol, tannin, gallic acid, catechin, pyrogallol, rutin, coumarin, trans-cinnamic acid and caffeic acid.

Histochemical reactions

It is well-known that anthocyan-type colour substances in the cell sap change their colour following alkaline treatment. For this reason 0.1 n sodium hydroxide was absorbed through cross section preparations to test if the colouring characteristic for phenols is visible. 12% potassium bichromate solution and 3% ferrichloride solution were used for histochemical reactions out of the tannin-(polyphenol)-reagents suggested by Petri (1979), Tyihák (1979) and Wagner et al. (1983). Reactions were achieved by the aqueous reagent solutions through microscopic preparations, between the slide and coverglass. Polyphenols form a reddish brown precipitate

with potassium bichromate, whereas they become greyish or greenish blue with ferrichloride.

According to our studies this method is suitable aspecifically for determining the tissue localization of phenoloids from the absorbed allelochemical substances.

Alkaloids were detected with Dragendorff reagent and Mayer reagent following Petri (1979). Localization of the absorbed atropine (and coalkaloids) and caffeine was best proved by microprecipitate formation with Mayer reagent.

Phytochemical studies

Chlorophyll content. 1.0 g out of the fresh, cut bean leaf samples was rubbed with 1 g quartz sand and a pinch of calcium carbonate in a friction cup with 10 ml acetone for 1 minute, then, after adding 0.50 ml petrolic ether and waiting for half a minute, rubbing was continued for another minute. Then the homogenizate was centrifuged at 3500 rpm for 10 minutes. After this the extracts were filled up to 10 ml with acetone. Absorption was measured with a Hitachi spectrophotometer at 665, 645 and 625 nm. Chlorophyll content was calculated by the traditional method, based on an empirical formula.

Thin layer chromatography and densitometry. Imbibition was carried out with 2 mM atropine, scopolamine, belladonnin, tropin and caffeine solutions. Alkaloid separation and identification were carried out from freshly cut leaves as follows: i) Tropane alkaloids: 1 g plant material was carefully homogenized with 1 ml ammonium hydroxide, then extracted with 30.0 ml chloroform, then shaken twice in a bolting funnel with 20 ml 2% sulphuric acid, then pH was set to 9–9.5 by adding 25% ammonium hydroxide. The chloroformic phase was filtered through a filter containing anhydrous sodium sulphate and evaporated in vacuum. The rest was dissolved in 5.00 ml chloroform. ii) Caffeine: 1 g plant material was extracted with 10 ml solvent mixture (5.5 ml 96% ethanol + 3.0 ml chloroform + 1.5 ml distilled water) on water bath using refluxing-cooler for 5 minutes and refilling with the solvent mixture to 5.5 ml.

Chromatographic plates: silica gel 60 F254 TLC (Merck, Darmstadt, Germany).

Sample application: with Minicaps 5, 10 µl and Hamilton syringe (Bonaduz, Switzerland).

Mobile phase: a) tropane alkaloids (Botz and Szabó 1988): 0.2 M NaAc in water + methanol + chloroform (1:6:3); b) caffeine: ethyl acetate + methanol + water (77:13:10).

Post-derivatization with modified Dragendorff's reagent: the layers were dried at 90 °C for 5 min, then cooled to room temperature and dipped into the modified Dragendorff's reagent for 1 sec, and dried in air at room temperature for 30 min.

Light source: tungsten lamp and mercury lamp.

Detection: a) tropane alkaloids: 660 nm; b) caffeine: 254 nm (without derivatization).

Densitometry: Camag TLC Scanner II (Muttenz, Switzerland) equipped with CATS software.

Scanning condition: slit dimension: 6 × 0.3 nm; monochromator band width: 30 nm.

For each measurement the leaves of 10 plants were used, separately picked from plants with roots and excised plants. There was no significant difference between weight measurements and densitometric measurements at p = 5%, which means that the method is reliable if precisely keeping the experimental conditions.

RESULTS AND EVALUATION

Histochemical studies

Imbibition experiments, whose aim was to prove the senescence-inducing effect of allelopathic, known substances, were carried out with bean plants grown and pretreated in the manner described in the methodological part.

First, however, imbibition with solutions was carried out. Intact and derooted bean plants with primary foliage leaves were placed into the aqueous solutions of allelochemical substances (juglone, catechine and tannin). As a control distilled water was used. One-day long imbibiton experiments were done with plants in the same developmental stage. Following this, plants were taken out of the solutions, rinsed with water and freshly processed for histological purposes. Cross sections were made out of the hypocotyl, the epicotyl, the petiole, and – in the case of plants having root – out of the tap root. In each case, cross sections made at the middle re-

gion of the mentioned stem- and root parts were used for histochemical reactions.

The histochemical reaction done with ferrichloride also proved that in the epicotyl of a rooted plant the presence of absorbed juglone is obvious. Compared with the control it can be seen well that in the smaller xylem parenchyma cells adjacent to the wide-lumen tracheas and tracheids juglone, reacting with a dark colour (dark brown) can be identified in a large amount. Xylem parenchyma cells formed during secondary thickening contain juglone similarly to the most important elements of the vascular tissue, tracheids and tracheas. It is even more characteristic for the derooted bean epicotyl, since absorption without root has no barriers, the allelopathic substance is present in the vascular system and the neighbouring parenchymatous tissue elements. Besides the ferrichloride reaction, the same fact is proved by the histochemical reaction with potassium bichromate.

In the tap root, for example, tannin can be found abundantly next to the vascular bundle and in the xylem parenchyma cells of the pith tissue bordering the stele. Tannin can be seen as a dark blue precipitate because of the characteristic ferrichloride reaction.

The fact that the easily absorbed catechin soon appears already in the hypocotyl – especially in derooted plants – can be verified also by a histochemical reaction with sodium hydroxide. It can be observed along the cambium as well that the presence of catechin, forming a dark greenish brown precipitate, is more intensive near vascular elements. This is even better supported by the reactions with potassium bichromate and ferrichloride, forming ample precipitate in the vascular system and neighbouring cells of the derooted hypocotyl. The presence of catechin in the epicotyl and the petiole is proved in the same way by the histochemical reaction with ferrichloride. It has to be noted that the absorption of tannin and catechin is also visible to the naked eye, since the solution streaming upwards in the stem causes a faint greyish or brownish colouring in the case of tannin and catechin, respectively, in the hypocotyl and epicotyl, as well as in the petiole and the main vein of the primary foliage leaf.

The studied alkaloids (atropine, caffeine) can be detected in the leaf tissue by Dragendorff and especially by Mayer reagent. Both atropine and caffeine is visible in the form of precipitate grains in the vascular elements, mainly in the parenchyma cells at the border of pith and primary cortex (Figs 1–3).

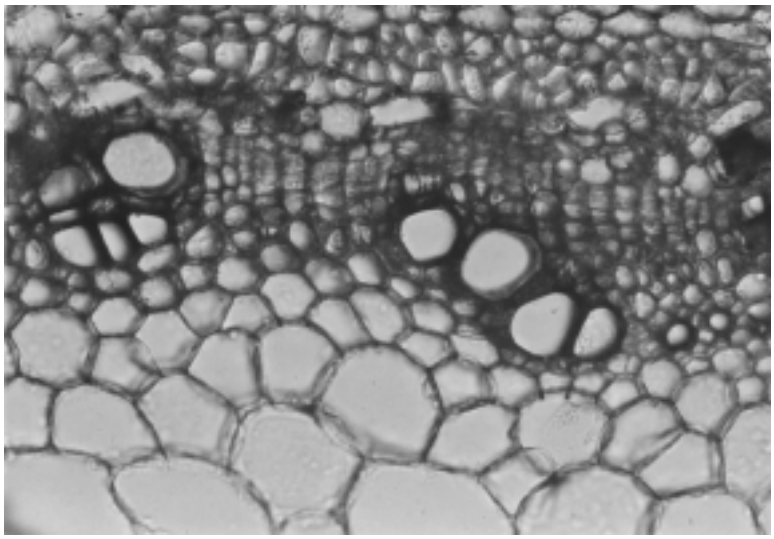

Fig. 1. Cross section of the bean leaf absorbed by 5 mM caffeine

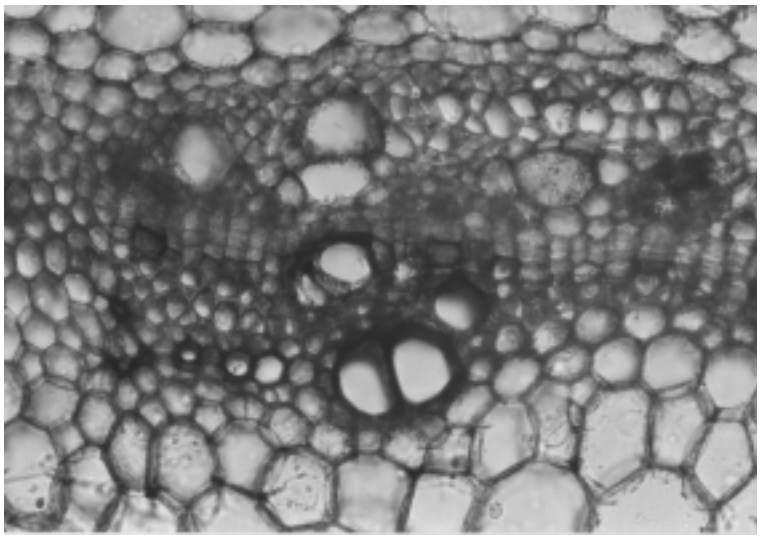

Fig. 2. Cross section of the bean leaf absorbed by 5 mM caffeine and reacted by Mayer reagent

Morphological changes, symptoms

Comparative studies were carried out at the same time. In the experiment, besides the distilled water control, imbibition was done with the

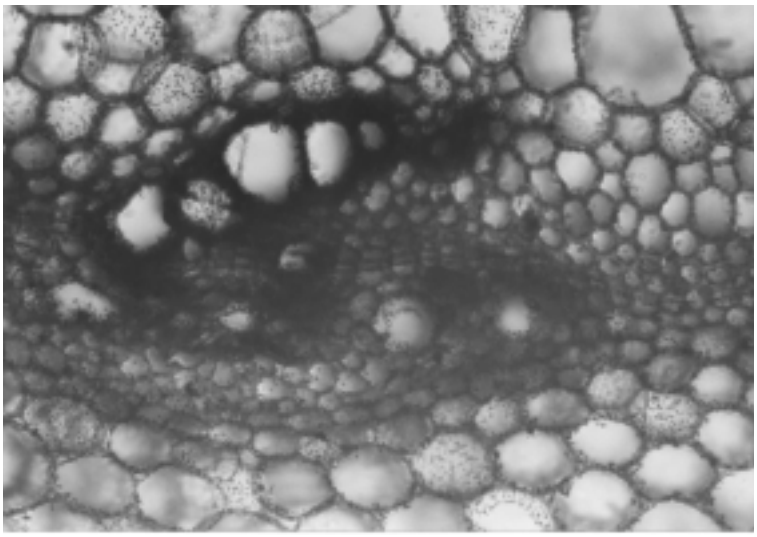

Fig. 3. Cross section of the bean leaf absorbed by 5 mM atropine and reacted by Mayer reagent

same concentrations of hydrochloric acid, sulphuric acid, citric acid, indol acetic acid and known allelochemical substances (juglone, coumarin, t-cinnamic acid, caffeic acid, gallic acid, catechin, tannin, pyrogallol, rutoside, thymol, atropine). In each case the pH values of the solutions was measured. Obviously, acids were characterized by a lower pH than substances with no acidic character. For that very reason, it proved to be important to compare the simple acidic effect with the "bioactive" effect, which supposedly has a different character. Testing the effect of solutions with relatively high concentrations (0.1% or 1,000 ppm) seemed to be reasonable, since allelochemical substances are present also in such concentrations in nature.

Control plants wilted on the 43rd day (at the end of the observation), but they flowered (1 or 2 flowers developed on each specimen) and in some cases even developed pods. Distilled water did not contain any kind of organic or inorganic nutritive material. Nutriments of the cotyledon and primary metabolites formed in the course of photosynthesis made this kind of development possible. Compared with this, every chemical intervention caused depressive symptoms. It has to be emphasized that cotyledons were kept in each case.

The fastest senescence and decay was caused by 0.1% hydrochloric acid, 0.1% sulphuric acid, 1,000 ppm indol acetic acid, 1,000 ppm coumarin, 1,000 ppm trans-cinnamic acid, 1,000 ppm gallic acid and 1,000 ppm

catechin. This dramatic effect is not surprising in any of these cases, since in a high concentration indol acetic acid is toxic, as well as coumarin or trans-cinnamic acid (which are known otherwise as toxic) or even gallic acid and catechin.

Wilting of leaves is frequently not accompanied by the total breaking down of chlorophyll, leaves wither and fall off almost green. Acidic effect is supposed to damage the vascular system (and the cambial region responsible for secondary thickening) in the same way as stronger allelochemical agents. The consequence of all these is transport disorder or total inhibition, which causes the quick wilting and drying. This is indicated also by the fact that the treatments induce also the early abscission of cotyledons. At higher concentrations only pyrogallol, juglone and tannin induce faster yellowing of leaves, whereas atropin causes leaf browning. 1,000 ppm juglone, caffeic acid, tannin, pyrogallol, thymol and atropine are also toxic, plants decay approximately on the 15th day.

Citric acid and rutin are the least toxic in high concentration. Citric acid takes part in metabolism in the known way, as a weak acid it is less tissue damaging. Plants wither gradually until the end of the observation period. Rutin causes total wilting on the 23rd day, but no drying.

A more differentiated effect is caused by less concentrated (0.01% or 100 ppm) solutions. 0.01% hydrochloric acid is more toxic and causes faster total decay than 0.01% sulphuric acid. The tissue damaging effect of the acid is also obvious from the fact that the adventive root formation – which is characteristic for bean and is pronounced at the control – takes place only in the region of the hypocotyl above the solution. On the impact of more concentrated solutions no root formation takes place, except for citric acid, where roots are formed above the solution. In the case of less concentrated citric acid root formation occurs in the solution, too, just like at the control. On the impact of 100 ppm indol acetic acid pronounced root formation can be observed above the solution, as far as the node of the cotyledon.

Among allelochemical agents at a 100 ppm concentration the most damaging is coumarin, followed by t-cinnamic acid, caffeic acid, pyrogallol, thymol and atropine. A slow, protracted decay is caused by juglone, gallic acid and catechin. The least toxic are tannin and rutin. From the latter, rather slowly damaging substances gallic acid, catechin, tannin and rutoside do not damage the already developed adventive roots even until total withering. Theoretically these remain in a turgid and living (but not further developing) stage as far as water uptake is ensured.

Thus the effect of allelochemical substances is highly varied and not uniform at all. The observation of developmental characteristics in the bean shoot having primary leaves and no root offers an excellent biotest possibility.

As already mentioned, degradation of chlorophyll does not seem to be very expressed on the impact of treatments. This supposition could be verified by measurements. Chlorophyll content of fresh primary foliage leaves from rooted and derooted shoots was measured on the 2nd day from the beginning of imbibition, i.e. after 48 hours. At this time no wilting symptoms can be seen on the plant. Chlorophyll content was measured in the way described at the applied methods (Fig. 4). It can be seen from the data that in the case of derooted plants chlorophyll content decreased to the smallest extent on the impact of tannin and to the highest degree on the impact of catechin. The inhibitory effect of the latter is not significant. As compared to the control, however, chlorophyll content decreased in all treatments. In the case of rooted plants it is obvious that a rather significant chlorophyll degradation was caused by the coumarin and then the juglone treatment. Trans-cinnamic acid and catechin decreased chlorophyll content to a lesser degree. Tannin proved to be the less effective.

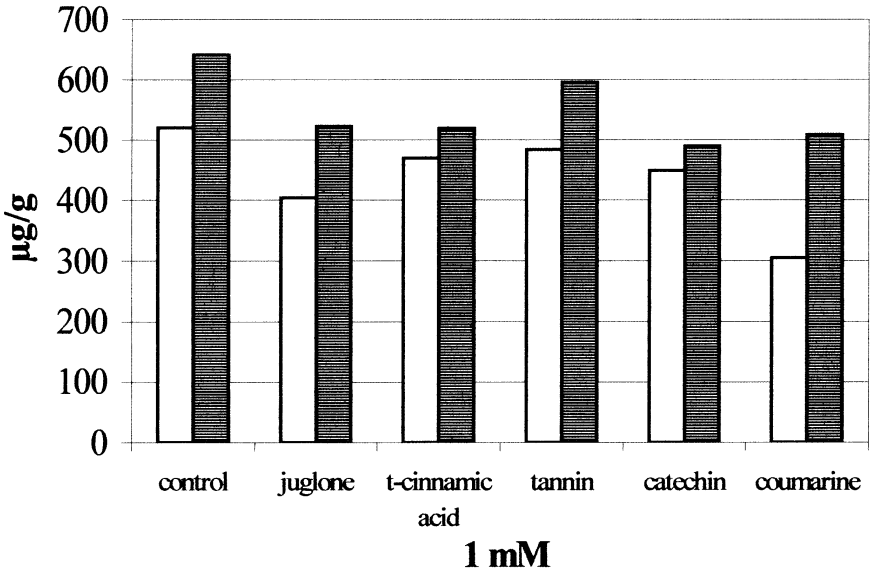

Fig. 4. Induced senescence by allelochemicals – chlorophyll content of the fresh primary bean leaves after 48 hours of the absorbing in 1 mM allelochemicals (intact = empty column; excised = hatched column)

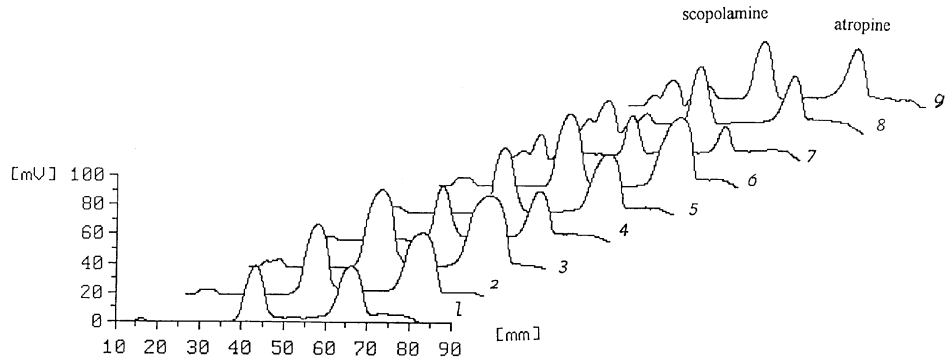

Fig. 5. Densitograms of tropane alkaloids absorbed by intact and excised bean leaves in 2 mM solution after 3 days (wavelength: 520 nm). 1: 5 µl, intact; 2: 10 µl, intact; 3: 15 µl, intact; 4: 5 µl, excised; 5: 10 µl, excised; 6: 15 µl, excised; 7: 2–2 µg tropane alkaloids; 8: 5–5 µg tropane alkaloids; 9: 7–7 µg tropane alkaloids

Thus it can be verified that in leaves showing no external symptoms, a smaller degree of chlorophyll degradation takes place already. The intensity of the impact can be supposed already at this stage, in a decreasing order of the inhibitory effect: coumarin, juglone, catechin, trans-cinnamic acid and tannin.

Phytochemical changes

From the studied tropane alkaloids the two main compounds, atropine and scopolamine could be measured with densitometry following

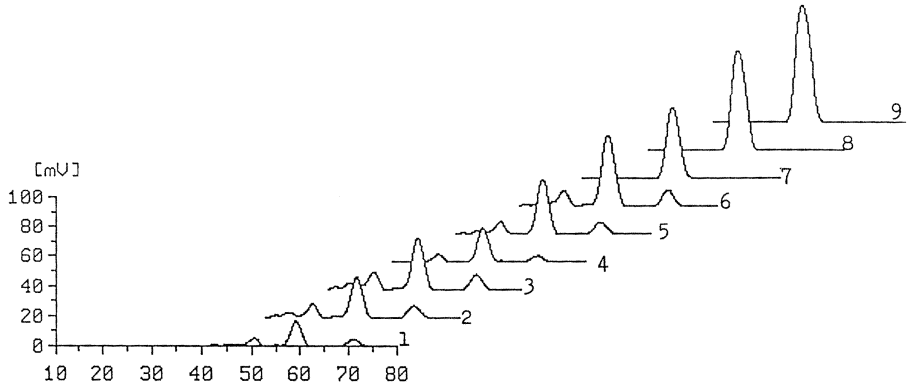

Fig. 6. Densitograms of caffeine absorbed by intact and excised bean leaves in 2 mM solution after 3 days (wavelength: 254 nm). 1: 5 µl, intact; 2: 10 µl, intact; 3: 15 µl, intact; 4: 5 µl, excised; 5: 10 µl, excised; 6: 15 µl, excised; 7: 2 µg caffeine; 8: 5 µg caffeine; 9: 7 µg caffeine

thin layer chromatography of bean leaf samples. From these samples neither tropin, nor belladonnin could be detected on the chromatogram after post-derivatization. This can be seen in the densitograms as compared with the control (Fig. 5). However, the amount of the already absorbed atropine and scopolamine can be measured quantitatively with densitome-

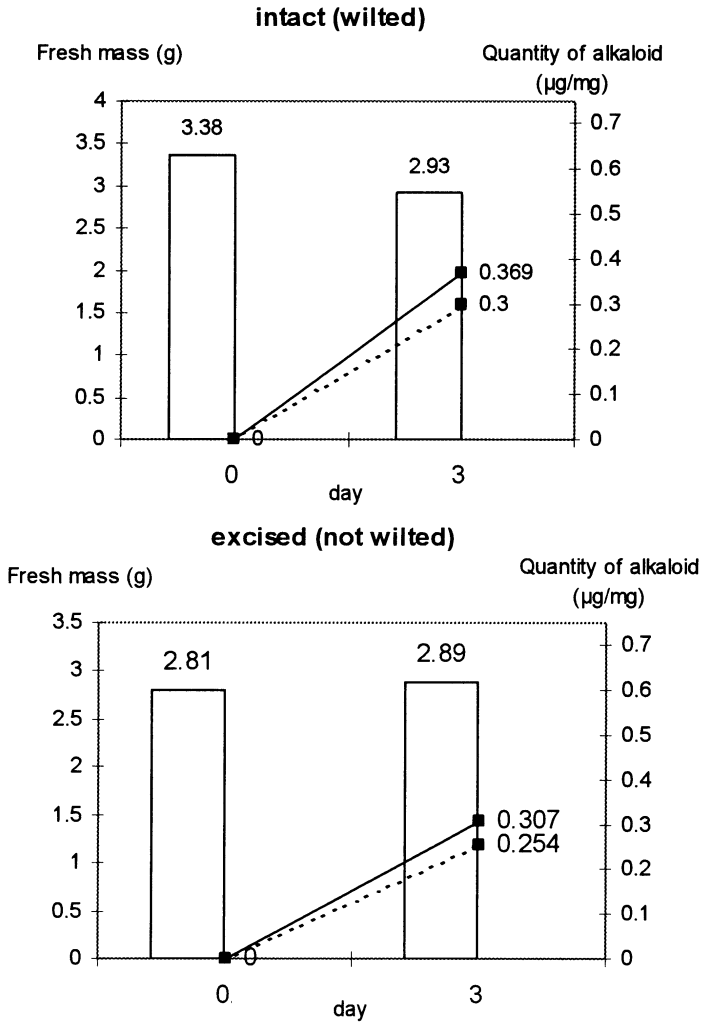

Fig. 7. Quantity (µg/mg) of atropine and scopolamine in bean leaves (fresh mass, in gramme) after 3-day absorbing of 2+2 mM solution (column = plant; atropine = dotted line; scopolamine = continuous line)

ter from already wilting bean leaves (Fig. 7), i. e. it can be detected (with regard to fresh weight):

- in the case of the intact, rooted and wilted plant: 8.6% of the available atropine (0.300 µg/mg from the 5 mM solution) and 16.8% of the available scopolamine (0.369 µg/mg from the 5 mM solution);
- in the case of the excised plant, not wilting yet: 7.3% of the available atropine (0.254 µg/mg from the 5 mM solution) and 14.0% of the available scopolamine (0.307 µg/mg from the 5 mM solution).

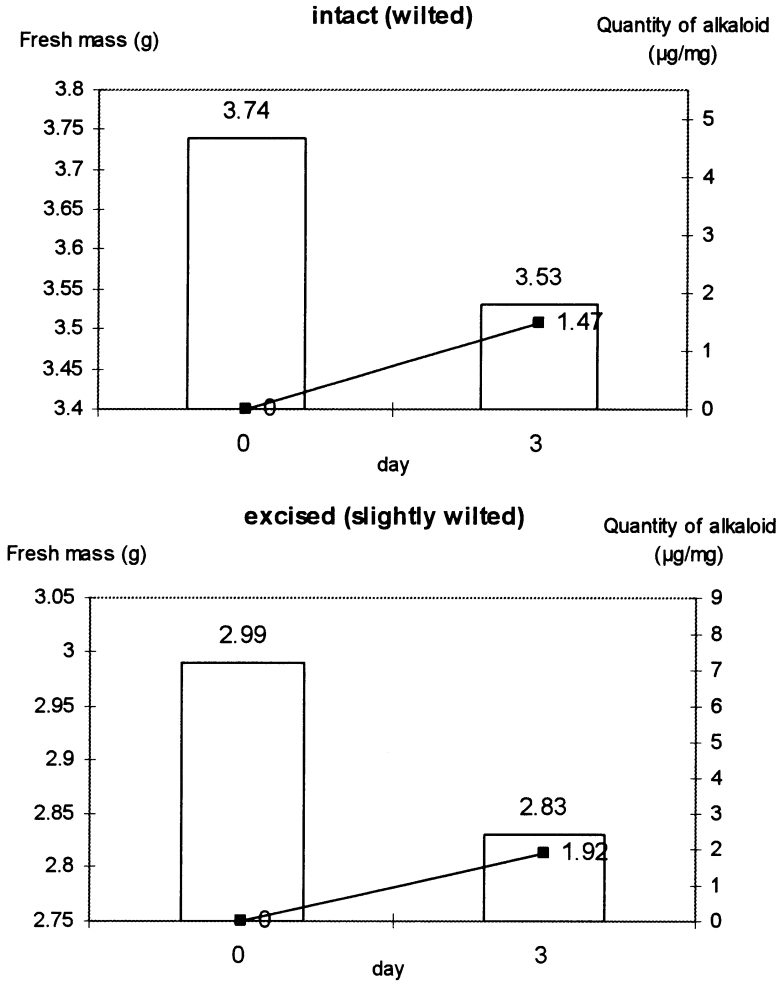

Fig. 8. Quantity (µg/mg) of caffeine in bean leaves (fresh mass, in gramme) after 3-day absorbing of 2 mM solution (column = plant; caffeine = continuous line)

The translocatory ability of caffeine, the studied purine alkaloid totally differs from the tropane alkaloids, despite of the fact that on the densitograms one can observe two unidentified derivatives so far. The absorbable main allelopathic effective substance, caffeine can also be measured quantitatively by densitometer both from intact (rooted) wilted and excised (derooted), slightly wilted bean leaves. Thus the amount of the absorbed caffeine can be determined (with regard to fresh weight):

– in the case of the intact, wilted plant: 151.5% of the available caffeine (1.47 µg/mg from the 5 mM solution);
– in the case of the excised, slightly withered plant: 198.0% of the available caffeine (1.92 µg/mg from the 5 mM solution).

As a consequence, only a small part of atropine and scopolamine can be identified in a decomposed form. This phenomenon was confirmed also by the positive histochemical reaction. The caffeine exerts its senescence inducing effect accumulated in the leaf tissue. It means that the absorption and translocation characteristics of alkaloids which seem to be more stable chemically may differ to a great extent.

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