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# A SIMPLE METHOD FOR VISUALIZATION OF PHENOLICS EXUDATION BY ROOTS OF INTACT LUPIN PLANTS; THE EFFECT OF NITRATE AND pH

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**Phenolics exudation by imbibed seeds and roots of intact lupin plants (*Lupinus albus* L.) was studied during the first 4 days of growth by a new agar test with specific reagents for phenolics (Gibbs reagent, Naturstoffreagenz A).**

**Comparative studies of the phenolics exudation reveal that legumes exude different phenolics (even if not qualitatively, then at least quantitatively) than oat. The exudation of phenolics starts very quickly after the imbibition of seeds and can be visualized as early as 24 h after sowing. In older seedlings, the exudation of phenolics can be detected along root zones and is influenced by nitrate and pH. At acidic pH, nitrate reduces phenolics exudation, but at pH 7.5 the exudation of phenolics becomes restricted to only some root zones. Nitrate must be present in the rooting media for at least 24 h to cause visible changes in the pattern of exudation at different pH values.**

The exudation of phenolics by plant roots is well known [1]. Phenolics were considered mainly as protective substances [2], e.g., allelopathic agents [3] or phytoalexins [4]. Recent findings reveal that they also act as

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Abbreviations: NA, Naturstoffreagenz A; t.l.c., thin-layer chromatography

chemical signals in plant-plant or plant-microbe systems (for review, see [5]), e.g. flavonoids and isoflavonoids, exuded by legume roots, are active in regulation of bacterial *nod* genes [6, 7]. There are also some data indicating localized signals for phenolic exudation by plant roots [8], but they were not consistently confirmed [9].

Nitrogen influences the formation and development of functional nodules [10], starting from the attachment of bacteria to the plant root surface and ending with the senescence of the mature nodule. To our knowledge, the data are not available on regulation by nitrogen of the signal for phenolics exudation. Thus, studies dealing with the plant "chemical signal processing" could provide an answer to this question.

For this purpose, we have developed a new method, utilizing specific reagents for phenolics, which enables observation, *in vivo*, of phenolics exudation.

## MATERIALS AND METHODS

*Plants.* Seeds of white lupin *(Lupinus albus* L. cv. Bac*)* were obtained from the Plant Breeding Station Przebędowo (Poland). Seeds of soybean (*Glycine max* L. Merr. cv. Progress) [11] and seradella *(Ornithopus sativus* L. cv. Mazurska Biała) – nodulated by *B. lupini* [12] – were from Dr J. Szukała from the Agriculture University, Poznań (Poland). Oat seeds (*Avena sativa* L. cv. Dragon), were purchased from Przedsiębiorstwo Nasiennictwa Ogrodniczego i Szkółkarstwa "CNOS" Poznań (Poland).

Soybean seeds containing isoflavonoids and the seeds devoid of isoflavonoids were used for comparative purposes.

*Chemicals.* 2,6-Dichloroquinone-4-chlorimide (Gibbs reagent), specific for 4-unsubstituted or 4-alkoxyphenols [13], was from E. Merck (Germany). Diphenylboric acid-β-aminoethyl ester (Naturstoffreagenz A, NA), specific for α- and γ-pyrones [14], was from Roth (Germany).

Samples of flavonoids (naringenin, apigenin, luteolin, kaempferol, quercetin and rutin) were a gift from Dr E. Kujawa (Institute of Medicinal Plants, Poznań, Poland). Isoflavonoids (genistein, 2′-hydroxy- genistein) and genistein glycoside of unknown sugar component from the white lupin roots were purified as previously described [15]. Phenolic acids (*m*- and *p*-hydroxybenzoic, *p*-methoxybenzoic, *o*-hydroxyphenylacetic and 3-phenylpropionic acids) were from Sigma (U.S.A.).

*Composition of nutrient media.* For preculture of plants and for agar plates, nutrient media (pH about 4.3), differing in the amount of nitrate, were prepared (in mM concentration): 1. "0 mM" solution: $CaSO_4$ – 1.7, KCl – 0.9, $KH_2PO_4$ – 0.55, $MgSO_4 \times 7H_2O$ – 0.3, $FeCl_3$ – 0.05; 2. "0.5 mM" solution: $Ca(NO_3)_2 \times 4H_2O$ – 0.36, $KNO_3$ – 0.17, $CaSO_4$ – 1.3, KCl – 0.7, $KH_2PO_4$ – 0.55, $MgSO_4 \times 7H_2O$ – 0.3, $FeCl_3$ – 0.05; 3. "9 mM" solution: $Ca(NO_3)_2 \times 4H_2O$ – 6.1, $KNO_3$ – 3.0, $KH_2PO_4$ – 0.55, $MgSO_4 \times 7H_2O$ – 0.3, $FeCl_3$ – 0.05.

For some agar tests on pH effects, pH of media was adjusted to 4.5 or 7.5 with diluted $H_2SO_4$ or KOH.

*Preculture of the plants.* Seeds of white lupin and oat were sterilized with 5% $H_2O_2$ (v/v) for 10 min and 3 min, respectively. Seeds of soybean and seradella were surface sterilized with 2.5% (w/v) of $HgCl_2$ for 2 min and 1min, respectively. After thorough washings with sterile distilled water, seeds were planted in trays on several layers of filter-paper moistened with sterile redistilled water and germinated in darkness in a temperature-controlled room at 25°C for up to 72 h. Three-days-old seedlings were transferred to dishes onto several layers of filter-paper moistened with one of the nutrient solutions and kept in darkness for 24 h at 25°C. At the end of this period, lupin and soybean roots were 30 – 45 mm long, oat roots were 40 – 55 mm and seradella roots were 10 – 20 mm long.

*Preparation of the test.* This method is based on that of pH measurement described by Weisenseel *et al.* [16] and modified by Marschner *et al.* [17].

Proper nutrient solutions were mixed with agar (0.75% w/v) and boiled. Three test plates were used for each experimental treatment. Two of them contained specific reagents (Gibbs reagent – 0.0125 % or NA – 0.01%; both w/v) and to the third plate, ethanol – solvent of the reagents at 0.5 % final concentration – was added as a control. Both the reagents and ethanol in these concentrations did not influence the growth of the seedlings.

Two seedlings of each plant species were placed in 11 cm Petri dishes in a way allowing maximal root embedding. To each dish, 40 ml of agar medium (36 – 38°C) was poured. Plant roots were oriented with pincers and then the agar was cooled to form a solid substrate. Dishes were kept in darkness for 5 – 6 h in a temperature-controlled room at 25°C.

All phenolic standards were dissolved in methanol (0.1%; w/v). Three test plates were prepared by mixing the "9 mM" nutrient solution with the proper reagent or with ethanol. In solid agar, holes (4 mm in diameter) were

made and 20 – 50 µl of standard solutions were poured into each well. Plates were incubated in identical conditions as for seedling growth.

*Visualization of phenolics exudation.* Plates containing Gibbs reagent were placed in a chamber saturated with diluted ammonia vapour (25 % $NH_4OH$ – $H_2O$, 1:4; v/v) and kept for 5 min. The picture with the best colour resolution was obtained during 3 – 20 min after ammonia treatment. $NH_4^+$ ions, present as nutrients in the medium, did not affect the specificity of the Gibbs reaction.

Control plates and plates containing NA were inspected under UV light (366 nm) and colours of the roots and root exudates were recorded. Then, plates were treated with ammonia vapour in the same manner as Gibbs plates and inspected under UV light (366 nm). Colour changes were recorded.

Plates with standards were analysed just as the experimental plates, depending on the reagent mixed with test media. The sensitivity of the method depends on the class of the compound. Isoflavonoid genistein becomes visible on Gibbs plates at the level above 1.5 µg, while, on NA plates, the amount of *m*-hydroxybenzoic acid must be as high as 10 µg to be visualized.

## RESULTS AND DISCUSSION

We propose a new simple method which enables direct observation of phenolics exudation by intact plant roots based on the use of two reagents specific for various classes of phenolics used in t.l.c. analysis: Gibbs reagent and Naturstoffreagenz A. However, the specificity of reagents is not equal for different compounds (Table 1).

### *Exudation of phenolics during the first 96 h of germination*

Every 24 h during the first four days of germination in water, lupin seedlings were tested for phenolics exudation on "9 mM" agar plates. The first observable change (24 h) with Gibbs reagent was the occurrence of a slight blue zone, ascribed mainly to phenolic acids, on and around the swollen seed. The zone later extended (48 h of growth; root length – 1 cm)

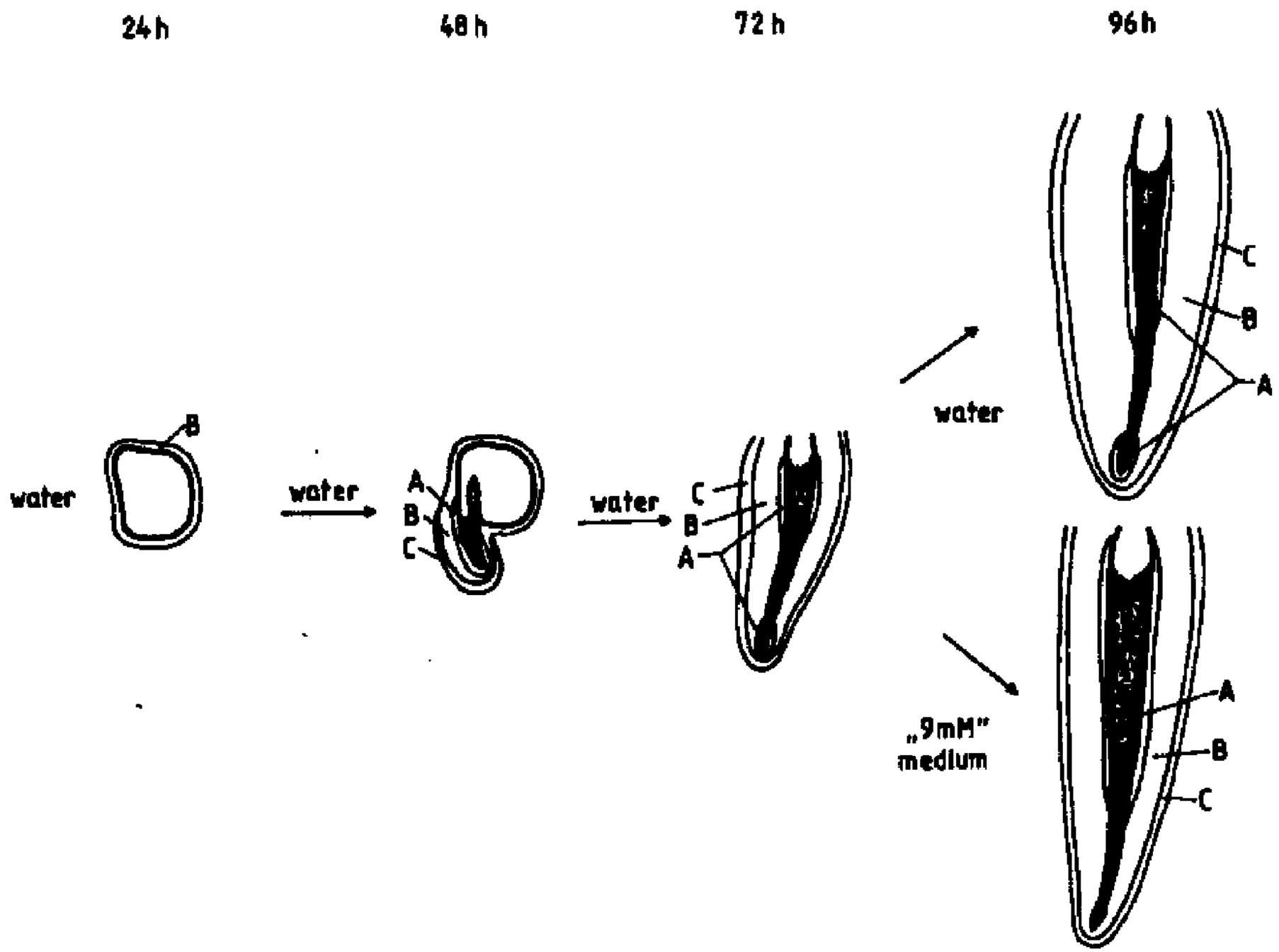


Fig. 1. Schematic presentation of phenolics exudation by lupin seedlings during the first four days of germination. Pictures were obtained in tests carried out every 24 h on "9 mM" plates with Gibbs reagents. Differences in exudation caused by preincubation of seedlings in the presence of nitrate are also indicated. A, brown zone; B, blue zone; C, purple zone

to 1 mm and still later (72 h of growth; root length: 2.5 – 3 cm) to a 4 mm area around the seed and the whole root. At 48 h of growth, two other zones appeared: a purple zone around the blue zone, and a brown zone (0.5 – 1 mm) around the meristem and the root hair zone (Fig. 1). The root surface darkening and the appearance of the brown zone should be ascribed to exudation of the compounds belonging to flavonoids, isoflavonoids and their glycosides. Lupin seedlings cultivated for four days revealed the same pattern of exudation as the 3-day old seedlings (Fig. 1).

These observations were confirmed by the use of NA reagent. Under UV light, various shades of yellow colour, observed around the meristem, can be attributed mainly to flavones and flavonols and their glycosides, which together with isoflavonoids, formed the dark brown zone along the roots. The formation of blue colours should be attributed to phenolic acids.

*Effect of nitrate on phenolics exudation*

Lupin seedlings, germinated for 72 h in water and then 24 h in water or nitrate media: "0 mM", "0.5 mM" and "9 mM", were tested on the same nutrient media. With the increase of nitrate concentration in the media containing Gibbs reagent, we observed a gradual decrease of phenolics exudation characterized by: 1) a decrease of the area of colour zones, and 2) diminishing and, finally, disappearance of blue and purple zones (see also Fig. 1). Only the brown zone was stable regardless of changes in nitrate concentration.

The lupin phenolics exudation was compared with that of other legumes (seradella and soybean) and oat. Patterns of exudation obtained on Gibbs plates were similar for all legumes (Fig. 1). The main common feature was the presence of a brown zone around the root hair zone and, to some extent, also above the meristem. In soybean and in seradella the blue and purple zones were less pronounced than in oat, in which, in contrast to legumes, the brown exudation zone was completely absent. Instead, a strong darkening of the meristems and a slight blue-purple zone were noted along the whole roots, more intense in the older parts of the roots.

The evident differences in root exudation between legumes and oat were confirmed by the observation under UV light (366 nm). In oat, these differences, mainly due to phenolic acids, were expressed by bright blue meristems (NA plate), light blue roots and a broad blue fluorescent zone

Table 1

*Colour reactions and the diffusion radius (in mm) of phenolic standards in the agar plates with two reagents specific for phenolics. Colour development after ammonia treatment is presented. For details see Materials and Methods. n.o. – not observed*

| Phenolics | Compound | Gibbs reagent | | NA | |
|---|---|---|---|---|---|
| | | colour | range | colour | range |
| Flavanon | naringenin | brown | 0.5 | n.o. | – |
| Flavones | aspigenin<br>luteolin | light blue | 0 | yellow<br>brown | 2<br>2 |
| Flavonols | kaempferol<br>quercetin | deep purple | 0.5<br>2 | light yellow<br>brown | 3<br>2 |
| Flavonol glycoside | rutin | grey | 2 | brown | 2 |
| Isoflavonoids | genistein<br>2′-hydroxygenistein | blue | 1 | brown | 0.5 |
| Isoflavonoids glucoside | genistein glycoside | blue-green | 2 | brown | 0.5 |
| Phenolic acids | *m*-OH benzoic<br>*p*-OH benzoic<br>*p*-$OCH_3$ benzoic<br>*o*-OH phenylacetic<br>3-phenylpropionic | bright blue<br>n.o.<br>n.o.<br>deep blue<br>n.o. | 6<br>–<br>–<br>6<br>– | fluorescent light blue<br>bright blue<br>bright blue<br>bright blue<br>bright blue | 5<br>4<br>3<br>3<br>3 |

around the roots (control and NA plates). As in the Gibbs tests, these blue zones were restricted to older parts of the root in the presence of nitrate.

Roots of the legumes studied revealed, under UV light, similar patterns of exudation. The root meristem was generally yellow (NA plate) and, after $NH_3$ treatment, this colour intensified and a yellow 1–2 mm zone appeared. The only differences between legume species were manifested by the intensity of the yellow zone along roots (NA plate) and in the colour of meristems (control plate) before and after $NH_3$ treatment. Thus, we concluded that, in the meristem zone, legumes exuded various phenolics belonging to the same class(es) of compounds.

We believe that differences in phenolics exudation are caused by the effect of nitrate on plant root metabolism. Previous observations of Marschner's group [17, 18] indicated that $NO_3^-$ uptake is both species and root zone specific and depends on pH of the environment. The observed localization of nitrate reductase activity in maize roots [19] is consistent with this picture. It is also known that the process of $NO_3^-$ assimilation in lupin is almost completely localized in roots [20]. The basic end product of $NO_3^-$ utilization is $OH^-$ ion [21]. The excess of $OH^-$ ions could lead to changes of internal root pH and also to a differentiated polarity of cell membranes in various root zones. On the other hand, some enzymes of the phenylpropanoid pathway are sensitive to changes of pH (e.g. phenylalanine ammonia-lyase [22], 4-coumaroyl-CoA 3-hydroxylase [23]). Thus, we conclude that this could lead to a differentiated synthesis of various phenolics and, in effect, to the changes observed in root exudation. It seems possible that changes in the phenolics exudation, ascribed to the effect of nitrate, can be one of the first effects of inhibitory activity of nitrogen in the legume-*Rhizobium* symbiosis (see also [7, 10]). However, conformation of this suggestion requires more precise quantitative analysis of particular phenolics in plant root exudates.

*Effect of pH on phenolics exudation*

Four experimental variants were prepared: "0 mM" and "9 mM" agar plates at pH 4.5 and at pH 7.5. Four-day old seedlings cultivated on sterile distilled water were tested on the "0 mM" plates, while plants which had grown for 24 h on the "9 mM" medium were tested on "9 mM" plates.

On Gibbs plates, at acidic pH, exudation under the influence of $NO_3^-$ was diminished. This was expressed by the shortening of the dark zone at the root surface, the disappearance of the brown zone around the meristem, and the weakening of blue and purple zones around the whole root. At pH 7.5, the blue zone was restricted to the root elongation zone and in "9 mM" plates, also to the zone around the stem base.

The brown zone observed at pH 4.5 and in a "0 mM" plate at pH 7.5, disappeared in the "9 mM" medium at pH 7.5.

Under UV light phenolics exudation (control and NA plates) by lupin roots was practically the same irrespective of the pH of the agar media used. The only exception was the appearance of a brown aureole around the root hair zone on the "9 mM" test plates.

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