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ROOT EXUDATES OF PLANTS

I. ANALYSIS OF ROOT EXUDATES OF BARLEY AND WHEAT IN THEIR INITIAL PHASES OF GROWTH

by V. VANČURA

Department of Soil Microbiology, Institute of Microbiology,
Czechoslovak Academy of Sciences, Prague 6

INTRODUCTION

The microflora of the rhizosphere differs qualitatively and quantitatively from that of the free soil mainly due to differences in the nutritional sources of the micro-organisms. While the nutritional sources of micro-organisms in a soil not influenced by the root systems of plants are mainly plant residues in various degrees of decomposition, micro-organisms in the rhizosphere receive nutrition by the so-called root exudates, which are mostly lower molecular weight substances exuded by the roots of growing plants. These substances, readily available to micro-organisms are of great importance for the population of the plant root surfaces and are from all aspects the determining factor of the rhizosphere effect. Knowledge on the composition of root exudates is consequently essential for the study of this phenomenon.

Data on various substances can be found in the literature [8 32 49 66]. Most studies have been devoted to the exudation of amino acids and sugars; other substances have been investigated only sporadically, sometimes even unrelated to the general composition of root exudates. Data on some groups are contained in the papers of Bhuvanesvari *et al.* [1 3], Rovira *et al.* [47–50], and Scheffer, Kickuth, and Visser [52 53]. A knowledge, as exhaustive as possible, of the complete composition of root exudates would help not only in an understanding of the mutual metabolic interaction between plants and micro-organisms but might also enable us to prepare

synthetic media suitable for the study of the relationships between soil micro-organisms and plant roots.

In this paper are reported the contents of the main carbonaceous and nitrogeneous, as well as of some physiologically active substances in the root exudates of barley and wheat in their initial growth phases.

MATERIALS AND METHODS

Preparation of the root exudates

Seeds of spring barley (*Hordeum distichon* L., variety "Stupický hanácký") and wheat (*Triticum vulgare* Vill., variety "Česká přesívka") were washed for two hours in running tap water followed by sterilization of their surfaces with 0.1 % mercuric chloride solution. Washing was then repeated with sterile distilled water and the seed then aseptically transferred to dishes with washed silica sand, wetted to 60% of its maximum water-holding capacity. After 7 to 9 days germination in the greenhouse the lids of the dishes were removed and the plants allowed to dry out to a degree which still permitted them to recover after wetting [29]. Re-wetting was carried out with sterile distilled water, and the plants were then left in this state for a period not exceeding 12 hours. The seed residues together with the upper parts of seedlings were then removed and the sand with roots washed on a Büchner funel with sterile distilled water, which was suctioned off. The solution containing the root exudate was centrifuged for 30 minutes (4,000 rpm), the supernatant decanted off, and the water removed by freeze-drying.

The resulting powder containing the root exudates was ground in an agate mortar and stored in a desiccator over potassium hydroxyde.

In order to check for possible contamination by extraneous organic matter, the same procedure was applied to non seeded sand.

Analysis of the root exudates

For the root exudates from barley and wheat obtained by the method described above the following data were determined: – Moisture (dry matter), inorganic substances (ash), reducing substances [39 57], total [35] as well as α-amino- and imino nitrogen [44]. Volatile acids were directly determined in the solution obtained after washing of the sand cultures prior to freeze-drying. All the other substances were estimated by chromatographic methods. Fifty to hundred milligrams of root exudates were dissolved in 10 to 20 ml distilled water and separated into substances adsorbed on cation exchangers, substances adsorbed on anion exchangers and neutral substances. Cations and amino acids were isolated on cation exchangers (Staionit FN in the H^+ cycle); these were eluted with 12% ammonia and the eluate evaporated in a current of warm air to dryness. The anions were adsorbed on the anion exchanger Dovex 1 in the carboxylic cycle [40]. Organic acids and inorganic acids were

eluted with 6*N* formic acid and 1*N* hydrochloric acid respectively. Both eluates and the solution containing the neutral substances were evaporated to dryness and kept in a desiccator.

Sugars were determined in the neutral fraction of the root exudates. An amount of evaporation residue corresponding to 100 mg root exudate was dissolved in 2 ml distilled water and 50–100 μl placed on Whatman I paper previously washed with distilled water. The chromatograms were successively developed three times in the following systems:– *n*-butanol/acetic acid/water (4 : 1 : 5), *n*-butanol/ethanol/water (4 : 1 : 5), *n*-butanol/pyridine/benzene/water (5 : 3 : 1 : 3), and in phenol saturated with water. Detection was carried out with silver nitrate [24], hydrogen aniline phthalate [42], with 0.2% resorcinol in *n*-butanol and with hydrochloric acid for ketoses, with Partridge's and Horrock's reagent [26] (ratio 14 : 1), with Riminis reagent for methylpentoses and with periodic acid and *p*-nitraniline for desoxysugars [25]. The oligosaccharides were eluted from the undetected chromatograms with water, the eluate evaporated to dryness and the dry residue hydrolyzed; partial hydrolysis was carried out in sealed ampoules with 0.12 *N* H_2SO_4 at 100°C for 10 minutes and complete hydrolysis with 1*N* H_2SO_4 at 100°C for 4 hours. After neutralization of the hydrolyzate with barium carbonate and removal of the precipitate, the sample was spotted onto the chromatogram, developed in the system *n*-butanol/pyridine/benzene/water (5 : 3 : 1 : 3) and finally detected with silver nitrate.

Volatile acids. Hundred millilitres of the solution containing root exudates from 200 plants were adjusted to pH 1.5 with 10 *N* H_2SO_4 and then steam distilled. Prior to the evaporation of the distillate to dryness in a current of warm air, it was titrated with 0.01*N* NaOH using phenolphthalein as indicator. The residue was converted to hydroxamates by the procedure described by Hais and Macek [25], and after dissolution in ethanol spotted onto the chromatogram. The chromatogram was developed in the system *n*-amylalcohol: acetic acid: water (4 : 1 : 5) and the spots detected with a 10% ferric chloride solution.

Hydroxy-di- and tricarboxylic acids were analysed after dissolving the residue obtained from the fraction adsorbed and eluted from the anion exchanger in 2 ml distilled water. The resulting chromatograms were developed in the systems *n*-butanol/formic acid/water (4 : 1 : 5) and ethanol/ammonia/water (90 : 5 : 5). Prior to detection the chromatograms were suspended for 48 hours in a current of air to permit the volatilization of formic acid or ammonia. Detection was carried out with bromphenol blue, with various modifications of the sugar–aniline reagent [51 56] and a 4% acetanhydric solution of *p*-di-methylaminobenzoldehyde [25].

The keto-acids were directly converted in the original root exudate solution to 2,4-dinitrophenylhydrazones [18]. After extraction, purification and evaporation to dryness at a temperature of 40°C, the sample was dissolved in 1 ml of a mixture of 10 parts of methyl alcohol to one part of pyridine. Fifty to hundred microlitres corresponding to from 5 to 10 mg of the exudate

to be analysed, were spotted onto Whatman paper No. 1 and developed in the system *n*-butanol/ethanol/water (4 : 1 : 5). The dinitrophenylhydrazones of keto-acids are coloured so that no subsequent detection is necessary. The colour, however, can be changed and made more intensive by spraying with 0.5*N* NaOH.

The amino-acids were determined by one-way and two-way paper chromatography in the fraction adsorbed on the cation exchanger; Whatman paper No. 1 was utilized previously washed with distilled water and with a 1% solution of the sodium salt of EDTA respectively. A quantity corresponding to 1.25–2.5 mg root exudate was spotted onto the paper and successively developed four to five times in the systems *n*-butanol/acetic acid/water (4 : 1 : 5). Tropeolin 000 served as the reagent indicating the course of the development. Detection was carried out by spraying with an 0.2% ethanolic solution of ninhydrin and subsequent heating for 10 to 15 minutes at 60°C. The final identification of the compounds was carried out by two-way chromatography. In the first direction the chromatograms were run overnight in the system phenol/ethanol/water (2 : 1 : 1) with 0.1% 8-oxychinoline, in the second direction in the system *n*-butanol/acetic acid/water (4 : 1 : 5) twice successively. Detection was carried out with an 0.4% ethanolic solution of ninhydrin and the chromatogram dried at room temperature.

For the determination of amino sugars three times the quantity of the sample was applied as for that of the amino acids in the same fraction. These compounds were detected with Elson's Morgan's reagent [19].

Derivates of indol. Two hundred milligrams of root exudate dissolved in 20 ml distilled water and the pH of the solution adjusted to 2.8–3.0 by means of 1*N* HCl. The resulting solution was then extracted three times with peroxide-free ether after two hours shaking in a shaking machine at 4°C in a dark room. The combined ethereal extracts were evaporated in a current of air to dryness and the evaporation residue dissolved in 1 ml water. The resulting solution was applied on Whatman paper No. 1. The chromatograms were developed in water saturated with butyl acetate and in the system isopropanol/ammonia/water (10 : 1 : 1). The compound were detected with Gordon and Weber's [23] modification of Salkovsky's reagent and with the formaldehyde reagent [45]. Detection was also carried out biologically using the cockle (*Agrostemma githago* L.) hypocotyl [9] in the eluate of the undetected spot.

Phenolic substances were isolated by ether extraction according to Smith [55]. The alkaline and acid ethereal extracts were separated, evaporated to dryness and the residue dissolved in 1 ml ethanol, up to 250 μl being placed on Whatman paper No. 1. Development and detection of the chromatograms was likewise carried out according to Smith [55].

RESULTS

The quantity of root exudate obtained by the above method of preparation corresponded to from 7 to 10 per cent of the dry matter of the parts of the plant growing above the soil. A single plant in its first ten days of cultivation in sterile sand liberates from its seed and exudes from its roots 0.4 to 0.5 mg substance into its surroundings. The root exudate in the freeze-dried state still contains about 8 per cent water. The dry matter of a typical batch of barley root exudates contained 19.1 per cent ash, 9.1 per cent reducing substances, 0.3 per cent volatile acids (determined before freeze-drying and calculated as acetic acid), 17.2 per cent non-volatile acids (calculated as malic acid) and 1 per cent total nitrogen of which 68.1 per cent were present as α-amino and imino nitrogen. Similar results were obtained for wheat root exudates.

Composition of the sugars

The best separation of sugars by partition chromatography was achieved with the system *n*-butanol/pyridine/benzene/water (5 : 3 : 1 : 3). Utilizing this system the following sugars were found in the root exudates of wheat (from the starting line to the front of the chromatogram): – four types of oligosaccharides, maltose, galactose, glucose, arabinose (fructose), xylose, ribose, and rhamnose: in the root exudates of barley, 3 to 4 types of oligosaccharides, maltose, galactose, glucose, arabinose (fructose), xylose, ribose, rhamnose, desoxyribose and another unidentified desoxysugar were found (Fig. 1). The amount of the individual components, expressed as a percentage of reducing substances for the system *n*-butanol/pyridine /benzene/water (5 : 3 : 1 : 3) is presented in Table 1.

The oligosaccharides obtained from the undetected chromatograms by elution were hydrolyzed and after neutralization subjected to chromatographic analysis. Glucose was found to be their main component; in addition, traces of galactose, arabinose, and xylose, and of arabinose and xylose were identified in barley and wheat respectively. On chromatograms developed in acid systems partial hydrolysis had already taken place with a resulting expansion of the corresponding spots (especially glucose).

Uronic acids were confirmed in barley and wheat root exudates by Tollen's, Neuberg's and Saneyoshi's test [27]. For further

TABLE 1

Sugars in root exudates of barley and wheat		
Compound	In % of reducing sugars	
	Barley	Wheat
Oligosaccharides	27.8	26.7
Maltose	5.4	3.1
Galactose	13.6	4.0
Glucose	9.5	16.8
Arabinose + fructose	19.0	17.7
Xylose	15.0	15.9
Ribose	1.3	0.9
Rhamnose	6.8	14.9
Desoxyribose	0.8	—
Desoxysugar	0.8	—

identification of the uronic acids, these were isolated in the form of barium uronates and allowed to react with thioglycolic acid in the presence of mannose [14]. The extinction curves of the reacting solution measured with a spectrophotometer indicated the presence of galacturonic acid in barley root exudates and of galacturonic and glucuronic acid in wheat root exudates. Because of the isolation conditions however, it was necessary to confirm these findings by further identification tests. The presence of uronic acids in barley and wheat root exudates was also proved by paper partition chromatography of the fraction isolated on the anion exchanger.

Among the amino sugars, glucosamine, or galactosamine (the compounds cannot be separated on a chromatogram) were detected in the amino acid fraction and in addition two unidentified amino sugars.

Organic acids

Keto-acids were detected in the root exudates after their conversion to hydrazones. The results are illustrated in Fig. 2. The hydrazones of keto-acids travel on the chromatogram as two spots. In the root exudates of barley and wheat two keto-acids *i.e.* pyruvic and oxalacetic acid were identified.

The hydroxy, di- and tricarboxylic acids found are presented in Figure 3 and comprise uronic acids, oxalic, malic, glycolic, succinic, and fumaric acid. Approximate relative ratios are given in Table 2.

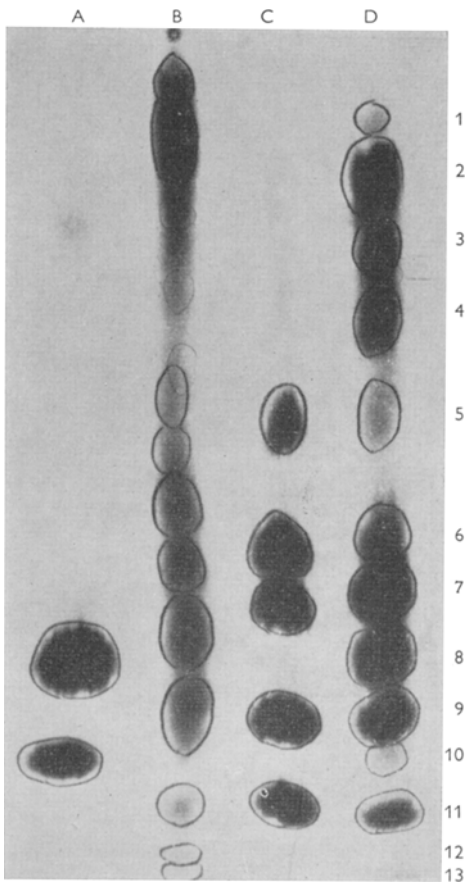

Fig. 1. Sugars in the root exudates of barley and wheat. **A**, **C** – standards, **B** – barley, **D** – wheat **1–4** oligosaccharides, **5** maltose, **6** galactose, **7** glucose, **8** arabinose, fructose, **9** xylose, **10** ribose, **11** rhamnose, **12** desoxyribose, **13** unidentified desoxysugar.

TABLE 2

Hydroxy-, di-, and tricarboxyl acids in root exudates of barley and wheat

Compound	Barley	Wheat
Oxalic acid	4	4
Malic acid.	3	3
Glycolic acid	3	3
Succinic acid	1	1
Fumaric acid	1	1

1–4 = area of the spots (traces to very large sports).

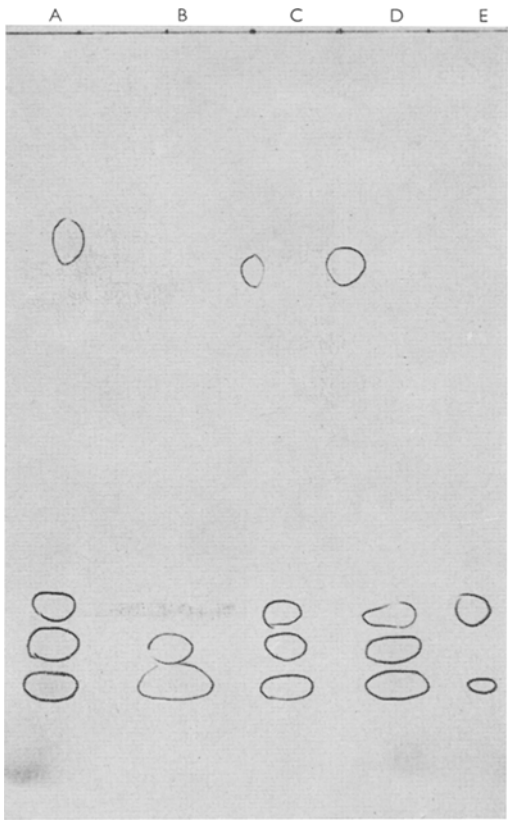

Fig. 2. Keto-acids in root exudates of barley and wheat. **A**, **D** – root exudates of wheat, **B** – pyruvic acid, **C** – root exudate of barley, **E** – oxalacetic acid.

Amino acids

The amino acids identified in barley and wheat root exudates and their approximate relative ratios are given in Table 3 and Figure 4; 14 and 18 amino acids were identified respectively. The barley root exudates contain cysteic acid and α-amino adipic acid besides the amino acids found in the wheat exudates; on the other hand cystine, glutamine, β-alanine, proline and γ-amino butyric acid were found in wheat exudates, but not in barley root exudates.

Derivatives of indol

In both plant exudates two growth promoting substances could be found. One of them, present in a higher concentration, could be

TABLE 3

Amino acids in root exudates of barley and wheat					
Compound	Barley	Wheat	Compound	Barley	Wheat
Cysteic acid	1	0	Threonine	3	3
Cystine	0	1	α-Alanine	3	3
Cystathionine	0	2	β-Alanine	0	1
Glutamine	0	1	Proline	1	1
Asparagine	3	2	γ-Aminobutyric acid	0	3
Aspartic acid	3	3	Tyrosine	3	2
Serine	2	3	Methionine (Valine)	3	3
Glycine	2	2	Phenylalanine	2	3
α-Aminoadipic acid	1	0	Isoleucine	2	3
Glutamic acid	3	3	Leucine	2	2

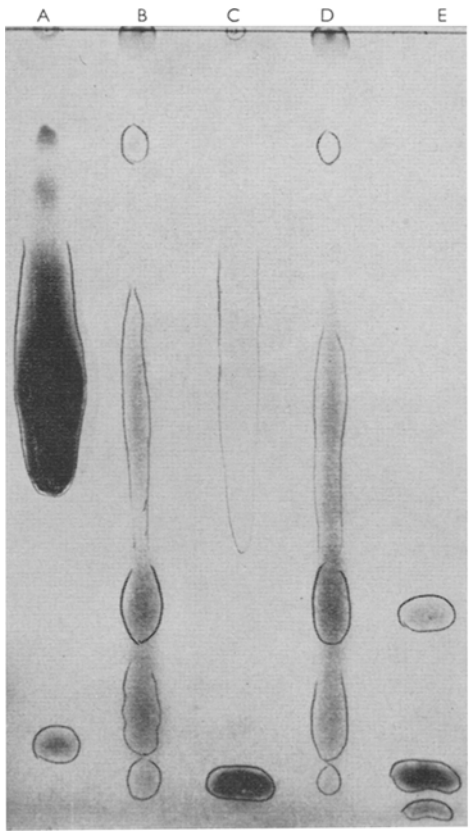

Fig. 3. Hydroxy- and dicarboxylic acids in root exudates of wheat (**B**) and barley (**D**). **1** – oxalic, phosphoric acid, **2** – malic acid, **3** – glycolic acid, **4** – lactic acid, **5** – succinic acid, **6** – fumaric acid, **7** – aconitic acid.

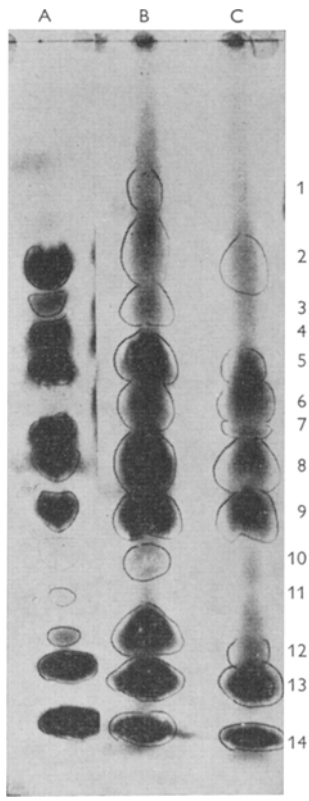

Fig. 4. Amino-acids in root exudates of barley and wheat. **A** – casamino-acids Difco, **B** – wheat, **C** – barley. **1–2** – cysteic acid, cystine, **3** – cystathionine, **4** – glutamine, **5** – asparagine, **6** – aspartic acid, serine, glycine, **7** – alpha-amino adipic acid, **8** – glutamic acid, threonine, **9** – α-alanine, β – alanine, **10** – proline, **11** – γ-aminobutyric acid, **12** – tyrosine, **13** – methionine, valine, **14** – phenylalanine, leucine, isoleucine.

identified as β-indolyl acetic acid (Fig. 5). The substance at the lower concentration could not be detected by chemical methods but was identified however, through its stimulation of the growth of the cockle hypocotyl. In addition to these compounds indol-3-carbonic acid was identified in both plant exudates and the barley root exudates also contained gramine.

Phenolic substances

In the alkaline ethereal extract of the root exudates of both

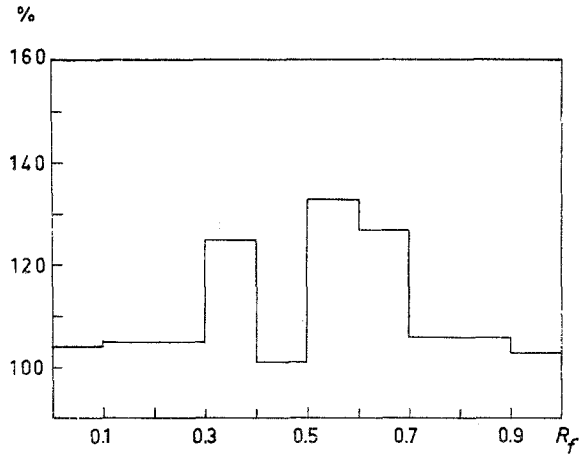

Fig. 5. Test on cockle hypocotyl of heteroauxin-type growth-promoting substances in barley root exudate.

Ordinate – percentage increase in growth; *abcissa* – R_f of spots in the system isopropyl alcohol/ammonia/water (10 : 1 : 1).

TABLE 4

Phenolic substances of non-acid character in root exudates of barley and wheat				
R_f isopropanol/ ammonia/water (20 : 1 : 2)	Fluorescence in UV	Spot colour after reaction with diazotized *p*-nitroaniline		Colour of the spot after reaction with ferric ammonium sulfate
			Na_2CO_3	
0.60	non fluorescent	orange	violet	after heating reddish brown
0.85	greenish blue	orange	scarlet	faintly reddish brown

TABLE 5

Phenolic acids in the root exudates of barley			
Spot no.	R_f *n*-butanol/ pyridine/water (14 : 3 : 3)	Colour of the spot after reaction with diazotized *p*-nitroaniline and sodium carbonate	Colour of the spot after reaction with sulphanilic acid and sodium carbonate
1	0.10	green	yellow
2	0.25	green	yellow
3	0.33	violet	brick red
4	0.52	violet	brown
5	0.56	yellow	yellow
6	0.64	yellow	violet
7	0.73	violet	violet
8	0.77	violet	orange yellow
9	0.88	violet	brown

plants, two spots corresponding to phenolic substances of a non-acid character are present (Table 4). From their reaction with ammonium ferric sulphate it can be concluded that first spot probably belongs to the group of mono- and meta-hydroxyphenols. With the system *n*-butanol/pyridine/water (14 : 3 : 3) eleven fluorescent spots were found in the acid ethereal extracts of barley and wheat exudates. Nine of the barley root exudates and seven of the wheat root exudates reacted with diazotized-*p*-nitroaniline. It might be concluded from the R_f values in various solvent systems and from the reaction with various detecting reagents [55] that ferulic acid, paraoxybenzoic acid, *o*-coumaric acid and also traces of gallic acid are present in barley root exudates. Ferulic and *o*-coumaric acid could also be found in wheat root exudates.

DISCUSSION

More and more evidence is accumulating in the literature that root exudates are utilized by a great number of micro-organisms. These exudates are essential for the rhizosphere effect and determine all related phenomena [30 46 48]. Root exudates obtained by the method described above have been found to promote the growth of Azotobacter [63] whilst amino acids, sugars as well as organic acids are utilized in various degrees by the bacterial associations isolated from root surfaces, from the rhizosphere and from free soil [62]. Fungal colonization of root surfaces is also related to root excretions [12].

In this work the method described by Katznelson, Rouatt and Payne [29 30] was used. These authors found that under conditions of desiccation plants excreted a greater amount of substances than when cultivated with adequate moisture supplies. In this connection it is not without interest that Clark [10] and Timonin [61] found the microbial population density higher at low than at optimum moisture content.

Since in our previous work [65] we analysed the root exudate of the same wheat variety, but grown in water culture, the composition found under these conditions can be compared with that of the root exudates of the same plant grown in a sand culture. The results show that there is no qualitative difference between these two cases. Differences were found in the relative distribution of the

individual components particularly of the hydroxy- di- and tricarboxylic acids and of the sugars. However, with the method of Katznelson *et al.* [29], the root exudates contain in addition substances released by germinating seeds, in contrast to the water-culture method, in which seedlings were used and the culture solutions changed. As shown by Balicka [2], Börner [6], Müller [38] and Pearson and Parkinson [43] amino acids and other substances are released from the swelling grain. The method of Katznelson *et al.* [29] enables one to obtain a substantially larger amount of root exudate than with a water culture or for instance, by root exudate absorption on activated charcoal as described by Scheffer, Kickut and Visser [52]. This applies especially to those cases where it is desirable to isolate a greater quantity of root exudates for microbiological purposes.

Most of the references available in literature on root exudates refer to the release of amino acids. Virtanen and Laine [64] investigated amino acid excretion from nodules of leguminous plants. The release of amino acids both from isolated and intact roots was later studied in other plants by Andal, Bhuvaneswari, Subba-Rao [1], Dehay and Carré [13], Frenzel [30], Fries and Forsman [21], Kandler [28], Katznelson, Rouatt, and Payne [30], Linskens and Knap [34], Martin [36], Parkinson [41], Pearson and Parkinson [43]; Rovira [47], Scheffer, Kickuth and Visser [52], Sulochana [59], Žák and Koloušek [67]. Amino acids in wheat root exudates have been studied by Katznelson, Rouatt, and Payne [30], Rivière [46] and Tesař and Kutáček [60]. The liberation of amino acids from swelling seeds of rye, barley, and wheat was investigated by Börner [6]. Most of the amino acids were identified by Tesar and Kutáček [60]; in addition to these we have found in the wheat root exudates, β-alanine, cystine and another not yet completely identified amino acid, which according to its position corresponds to cystathionine. Hydroxypipecolinic acid as reported by Rivière [46] could, however, not be detected.

The nature of the amino acids in barley root exudate was studied by Katznelson, Rouatt, and Payne [30] and Lasik [33]. In addition to those identified by these authors we found cysteic acid, serine, α-amino adipic acid, threonine, phenylalanine, and isoleucine.

The presence of sugars in root exudates was reported as long as 50 years ago [37]. More recently they have been found and their

composition in various plants investigated by Martin [36], Rovira [47], Scheffer, Kickuth, and Visser [53] and Schroth and Snyder [54].

The exudation of sugars by wheat roots has been studied by Duchoň, Kutáček and Tesař [15], Katznelson, Rouatt, and Payne [29] and Rivière [46]. Using suitable partition systems, detection reagents and other methods, it has now been possible to add to the list another oligosaccharide, maltose, galactose, ribose, uronic acids, glucosamine, two other not yet closely identified amino sugars, and certain other sugars.

The presence of glucose in barley root exudate was reported by Katznelson, Rouatt, and Payne [30], whilst in a medium containing swelling seeds of barley Börner [6] identified glucose and xylose and considered the presence of fructose as also probable.

In this work the presence of the following sugars and sugar like substances has been confirmed: – oligosaccharides, maltose, galactose, glucose, arabinose, fructose, xylose, ribose, rhamnose, desoxyribose and another not yet completely identified desoxysugar, glucosamine, uronic acids, and two other unidentified sugars.

Proof of the presence of organic acids in root exudates was also given many years ago [11 58]. There are, however, few references to the identification of the individual organic acids even in the most recent literature.

The identification of organic acids in the root exudate of *Lupinus luteus* was attempted by Fuss [22], who found six keto-acids and identified pyruvic acid. Dehay and Carré [13] found in wheat root exudates carbonic and citric acid. In the present work, besides a number of inorganic acids, oxalic, malic, glycolic, succinic, and fumaric acid have been identified in barley and wheat root exudates. No citric acid could be found although the systems and detecting reagents used allow for a convincing identification of this acid. Among the keto-acids pyruvic and oxalacetic acid were found.

The exudation of β-indolylacetic acid from the roots of peas was described by Žák and Kaloušek [67]. As far as phenolic substances are concerned the exudation of scopoletine and the β-glycoside of scopoletine has been reported for the roots of oats [16 17] and that of trans-cinnamic acid for the roots of *Parthenium argentatum* [4 5]. There is also a certain amount of information on the release of phenolic substances from germinating grains. Börner [7] reports the exudation of a glycoside from swelling seeds of flax which on

hydrolysis gave ferulic, *p*-coumaric and a hydroxybenzoic acid besides the aglucon. Knapp [31] found in the root exudates of germinating seeds of *Trifolium repens* and *Trifolium hybridum* the flavones quercetine and myricetine and myricetine respectively.

The active factor in root exudates of the grass *Eragrostis curvula* (Schrad) Nees, which is resistant to nematodes, was identified by Scheffer, Kickuth, and Visser [53] as pyrocatechol.

In the present experiments on the root exudates of barley and wheat a number of phenolic acids and certain other phenolic derivatives were found and further research will be devoted to their exact identification.

SUMMARY

The composition of the root exudates of barley and wheat in the initial growth phases was investigated; amino acids, organic acids, sugars and certain aromatic compounds could be identified. A knowledge of the composition of root exudates is important from the standpoint of the interaction between the plants and the micro-organisms in the rhizosphere. Some aspects of the rhizosphere effect connected with the present work are discussed.

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