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METHODS OF PLANT ROOT EXUDATES ANALYSIS: A REVIEW

P. Dundek, L. Holík, T. Rohlík, L. Hromádko, V. Vranová, K. Rejšek, P. Formánek

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Abstract

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The aim of this review is to summarise current knowledge on methods being used to determine individual compounds and properties of water-soluble plant root exudates. These compounds include amino acids, organic acids and simple sugars, as well as polysaccharides, proteins and organic substances. Qualitative composition of water-soluble root exudates and exudation rate are commonly measured with the aim of consequent synthetic preparation of plant root exudates to be supplied to soil to create artificial rhizosphere for different experimental purposes. Root exudates collection usually requires consequent filtration or centrifugation to remove solids, root detritus and microbial cell debris, and consequent concentration using an evaporator, lyophilizator or ultrafiltration. Methods used for analysis of total groups of compounds (total proteins and total carbohydrates) and total organic carbon are simple. On the other hand, HPLC or GS/MS are commonly used to analyse individual low molecular weight organic molecules (sugars, organic acids and amino acids) with separation using different columns. Other properties such as pH, conductivity or activity of different enzymes as well as gel electrophoresis of proteins are sometimes assessed. All of these methods are discussed in this work.

amino acids, HPLC, GC/MS, organic acids, root exudates, sugars

Substances released by healthy and intact roots into the environment are collectively designated as root exudates. These substances affect the composition of microbial community in immediate proximity of plant roots and processes taking place in rhizosphere. Many compounds are released by plant roots, including inorganic ions and substances, amino acids, amino acids, amides, sugars, aliphatic acids, aromatic acids, volatile aromatic compounds, gases such as ethylene, vitamins, peptides, proteins, enzymes, plant hormones, alcohols, ketones, olefins, urea, phytoalexins (Vančura, 1988; Grayston *et al.*, 1996; Paynel *et al.*, 2001; Aulakh *et al.*, 2001; Uren, 2007; Neumann and Römheld, 2007). In total, 200 different carbon containing molecules are present in root exudates (Curl and Truelove, 1986), and up to 40% of the net carbon fixed during photosynthesis can be released into the rhizosphere (Whipps and Lynch, 1990). These compounds need to be analysed using different methodological procedures. In this article we present the review of current methods used for pre-analysis preparation and analysis of different compounds which occur in plant root exudates.

Analysis of plant root exudates

Collection of root exudates

Water-soluble root exudates in collection solutions usually require subsequent concentration on an evaporator or lyophilizator or using ultrafiltration due to low concentration of certain exudate compounds. Not all analysis following exudates collection requires concentrated samples (e. g. total organic carbon or total carbohydrates). Prior to concentration or direct analysis, solids, microorganisms, and root border cells in collected solution should be removed by filtration or centrifugation steps. Depending on the composition of the collection solution, the reduction of sample volume can lead to high salt concentrations,

which may interfere with subsequent analysis, or may even cause irreversible precipitation of certain exudates compounds (e.g. Ca-citrate, Ca-oxalate, proteins). Therefore, if possible, removal of interfering salts using ion-exchange resins prior to sample concentration is recommended (Neumann and Römheld, 2007). Alternatively, solid-phase extraction techniques may be employed for the enrichment of exudates compounds from the diluted trap solution (Johnson *et al*., 1996; Gerke *et al*., 1994). High-molecular-weight (HMW) compounds may be concentrated by precipitation with organic solvents (methanol, ethanol, acetone 80% [v/v] for polysaccharides and proteins) or acidification (trichloroacetic acid 10% [w/v], perchloric acid 5% [w/v] for proteins (Neuman *et al*., 1999). Alternatively, ultrafiltration of the collection solutions or even cultivation of plant roots enclosed in dialysis bags is possible (Sakai and Tadano, 1993).

Analysis of low molecular weight compounds

Before the analysis of concentrated root exudates can be performed separation of exudates into acid, neutral and basic fractions using ion-exchange chromatography followed by separation of organic compounds in each fraction by e.g. HPLC (Schwab *et al*., 1983; Gransee and Wittenmayer, 2000; Gransee, 2001). Concerning HPLC analysis, Gransee and Wittenmayer (2000) used Bio-Sil NH_2 column (Bio-Rad, München), 150 × 4.6 mm, 5 µm particle size: eluent acetonitrile: water, 85:15 v/v isocratic at 30 °C, flux rate 1.5 mL. min^{-1} for separation of neutral compounds; Schwab *et al*. (1983) used conversion into trimethylsilyl derivatives using Tri-Sil reagent and separated individual carbohydrate derivatives on a GLC fitted with a 200 cm glass column packed with 3% OV-210 on 80- to 100-mesh Chromsorb; Schwab *et al*. (1983) used paper chromatography of underivatized samples of previously separated neutral fraction of root exudates to identify the sugars, using ethylacetate, pyridine and water (8:2:1, v/v/v) as the solvent and 5% *p*-anisidine as the developing agent. An Aminex 87H column, (Bio-Rad, München), 150 × 1.5 mm, 5 µm particle size: eluent 0.005 M sulphuric acid at 30 °C, flux rate 0.6 mL. min^{-1}, or a Bio-rad HPX-87 column, with 0.005 M H_2SO_4 following at a rate o 0.5 mL. min^{-1} at ambient temperature as the solvent (UV detection at 210 nm) or mobile phase 0.005 M H_2SO_4, 0.5 mL.min^{-1}, 50 °C, wavelength 210 nm were used for separation of organic acids (Schwab *et al*., 1983; Gransee and Wittenmayer, 2000; Wang *et al*., 2006); basic compounds were separated after precolumn derivatization with *o*-phtaldialdehyde (OPA) reagent on a RP select-B (Merck, Darmstadt), 250 mm × 4 mm, particle size 4 µm, gradient elution with 1 M TBAH in MeOH/MeCN/water 7.5:50: 442.5 → 7.5:350:142.5 (m/m/m), flux rate 1.0 mL.min^{-1} (Gransee and Wittenmayer, 2000). Nardi *et al*. (2002, 2005) used anionic fraction of root exudates to be separated by HPLC on an Aminex column (HPX 87 H – Biorad) with 6 mM H_2SO_4 as mobile phase and detection at 210 nm.

Liu *et al*. (2004) analyzed organic acids using HPLC with an ion-exclusion column (Shim-pack SCR-102h, 0.8 cm × 30 cm). The column was eluted for 30 min. at 23 °C with the mobile phase KH_2PO_4 (18 mM, pH 2.18); the flow rate was 0.8 mL.min^{-1}; organic acids were detected at 214 nm and a reversed-phase column C-18 (Silicon 250mm × 5.6 mm, Shimadzu, Japan) was used. Aulakh *et al*. (2001) analysed organic acids in root exudates according to modified protocol of Badoud and Pratz (1986) when lyophilized exudates were dissolved in distilled water, and derivatized by 1, 4 – dioxan containing O-(4-nitrobenzyl)-N, N-diisopropylisourea. After derivatization at 80 °C for 60 min., the samples were cooled at room temperature and 400 µL H_2O and 600 µL acetonitrile were added. Subsequently, 100 mg Dowex 50 W–X8 (100–200 mesh, p.a.) were added, and the samples were shaken for 2 min., incubated for 15 min at room temperature and centrifuged. The analyse by HPLC was performed using acetonitrile/water gradient (0–20 min, 20–80% acetonitrile) at a flow rate of 1 mL.min^{-1}, separation was performed on a reversed-phase octadecyl-silicium column, C-18, 5 µL, 250 × 4.6 mm (Ultrasphere™, Beckman, Germany) with detection at 265 nm. Ma and Miyasaka (1998) searched for oxalate, succinate, malate and citrate in root exudates of taro using HPLC with an anion self-regenerating supressor (4 mm, model ASRS-I, Dionex). For oxalate and other organic acids analysis Ma and Miyasaka (1998) used an anionic-exchange analytical column and a guard column (both 4 mm, models IonPac AS4A-SC and AG4A-SC, Dionex) with an eluent of 22 mM sodium borate and boric acid at a flow rate of 2.0 mL.min^{-1}. The analyse was reconfirmed by the authors using 50 mM NaOH as the eluent for oxalate, succinate, malate, and using 100 mM NaOH for citrate; concentration of organic acids was detected using a conductivity detector. George *et al*. (2002) extracted organic acids from rhizosphere soil by shaking the soil in deionised water with anion exchange strip (recharged with 0.5 M $NaHCO_3$). After cleaning the soil, elution of organic acids from the strip using 0.5 M HCl, protonation of organic acids by H_2SO_4, consequent methylation with methanol, cooling, application of water, chloroform and shaking, GS/MS analysis of organic acids was used (a Restek Rtx-50 capillary column, 5 m×0.25 mm i.d., film thickness 0.1 µm, connected to a Hewlett Packard HP 5970 mass spectrum detector, helium as the carrier gas). Vonderheide *et al*. (2006) measured selenium containing amino acids or inorganic compounds using HPLC-ICP-MS. The authors measured selenosulfate $SSeO_3^{-2}$ in root exudates using anion-exchange chromatography. Phillips *et al*. (2004) measured amino acids in root solutions by HPLC analysis of fluorescent derivatives produced by reaction with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Cohen and Michaud, 1993). Formánek *et al*. (unpublished)

measured 17 amino acids in root exudates using precolumn derivatization with *o*-phthalaldehyde (OPA). The measurement was performed using an HP 1100 liquid chromatograph (Hewlett Packard, Wilmington, DE, USA) with fluorometric detector FLD HP 1100 operating at 450 nm (Ex = 340 nm) with separation using a Zorbax Exlipse AAA Rapid Resolution (4.6 × 150 mm, 3.5 µm particle sizes, Agilent Technologies, USA). A linear gradient profile of mobile phase consisted of 40 mM Na_2HPO_4, pH 7.8 (solvent A) and ACN/MeOH/water 45:45:10 (v/v) (solvent B), 0% B (0–1.9 min), 0–57% (1.9–18.1 min), 57–100% (18.1–18.8 min), 100% (18.8–22.3 min), 100–0% (22.3–23.2 min) and 0% (23.2–26 min) was applied at a flow rate of 2.0 mL.min^{-1} (Formánek *et al*., 2005). Sometimes, for analysis of amino acids was used Amino Acids analyzer – column elution with sodium citrate buffers (Schwab *et al*., 1983; Bacilio-Jiménez *et al*.. 2003; Wang *et al*., 2006). Paynel *et al*. (2001) measured amino acids in root exudates using HPLC as *o*-phthaldialdehyde derivatives on a C–18 column using Gold system 8.0. Kravchenko *et al*. (1993; 2004) measured tryptophane in root exudates using HPLC with LiCrosorb RP–18 reversed phase column, which was eluted with solution containing 13.7% acetonitrile and 0.22% acetic acid in water. Wang *et al*. (2006) separated and quantified sugars in root exudates using in HPLC with an RI x 4 detector and Sugar-pak 1. P/N 85188 column (temperature 85 °C, mobile phase Milli-Q water, 0.6 mL.min.$^{-1}$). Bacilio-Jiménez *et al*. (2003) determined carbohydrates in root exudates with the heptafluorobutyrate derivatives of *O*-methyl-glycosides (after methanolysis in 0.5 M methanol-HCl) by gas chromatography, using a capillary column (25 × 0.32 mm) of 5% silicone OV 210 (gas chromatograph with flame detector, glass solid injector, carrier gas – helium). Erickson *et al*. (2001) used GS/MS for analysis of hydrofobic root exudates, Nardi *et al*. (2005) derivatized root exudates using 2,2-dimethoxypropane *plus* concentrated HCl to obtain corresponding methyl esters; after drying under N flow followed by supplementation of methanol, analysis was done using GS/MS (chromatographic separation using capillary column HP 50, 30 m, film thickness 0.5 µm, internal dia. 0.25).

Other types of analysis

Exudation all of the components released in soil can be suitably evaluated by the amount of carbon (Mench, 1985). Total organic carbon (TOC) is usually determined by wet digestion method (Nelson and Sommers, 1996; Aulakh *et al*., 2001), or using an analyzer (Dousset *et al*., 2001; Personeni *et al*., 2007; Miya and Firestone, 2001). Sometimes, content of nitrogen (Kjeldahl) is measured (Miya and Firestone, 2001; Nardi *et al*., 2002). Sometimes dissolved inorganic CO_2 is evacuated from collection media using HCl before the determination of TOC (Personeni *et al*., 2007). Analysis of total carbohydrates content in root exudates was performed using the anthrone colorimetric assay without lyophilisation of samples (Brink *et al*., 1960; Aulakh *et al*., 2001; Formánek *et al*., unpublished), Miya and Forestone (2001) used phenol-sulphuric acid method to estimate carbohydrate content (Ashwell, 1966; Dubois *et al*., 1956). Sometimes, exudates are concentrated and carbohydrates in neutral fraction separated from basic and acidic fractions (Schwab *et al*., 1983). Total soluble sugar content was also determined by colorimetric procedure using sulphonated α-naphthol (Denvor, 1950). For measurement of total sugars Wang *et al*. (2006) hydrolyzed concentrated root exudates with H_2SO_4 under vacuum at 110 °C; after cooling, filtration, drying and dissolution in 0.005 M H_2SO_4, the total sugars were separated and quantified using HPLC. Total amino acids were assessed e.g. by colorimetric procedure using ninhydrin (Spies, 1957); sample of root exudates before analysis by HPLC or amino acid analyzer can also be hydrolysed (Bacilio-Jiménez *et al*., 2003; Wang *et al*., 2006) e.g. by 6 M HCl under vacuum at 110 °C and with a drop of phenol to avoid degradation of tyrosine residues as reported by Bacilio-Jiménez *et al*. (2003). Miya and Firestone (2001) determined amino acids in root exudates using a meso-diaminopimelic acid assay (Daniels *et al*., 1994). Total proteins are mostly determined using Bradford's method (1976) (Bacilio-Jiménez *et al*., 2003; Nóbrega *et al*., 2005; Formánek, unpublished). Except frequently measured organic compounds, pH of the rhizosphere or conductivity of root exudates is sometimes measured. Wang *et al*. (2006) measured conductivity of root exudates at room temperature and after being exposed to high temperature, and from these values they calculated the percentage of electrolytes osmosized from roots. Activity of different enzymes (haem-containing peroxidases, unspecific copper-containing oxidases, unspecific haem-containing oxidase, flavin-containing monooxygenases – hydroxylating, aromatic-ring cleavage dioxygenases, oxydases that catalyse H_2O_2 formation and bleaching of dye macromolecules) or gel electrophoresis of proteins is sometimes assessed (Gramss and Rudeschko, 1998).

CONCLUSION

Determination of three most abundant compounds in water-soluble plant root exudates (sugars, organic acids and amino acids) is highly needed for various purposes. These compounds (even dominant within root exudates) occur in very low concentrations and sensitive analytical procedures

are necessary for their determination. Different approaches with the use of HPLC or GS/MS are utilized to determine individual sugars, organic acids and amino acids in plant root exudates. Determination of other properties of plant root exudates (concentration of total carbohydrates, proteins, pH, conductivity or activity of different enzymes as well as gel electrophoresis of proteins) is sometimes used.

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Address

Ing. Peter Dundek, Bc. Ladislav Holík, Bc. Tomáš Rohlík, Ing. Ladislav Hromádko, Ing. Valerie Vranová, Ph.D., doc. Ing. Klement Rejšek, CSc., doc. RNDr. Pavel Formánek, Ph.D., Ústav geologie a pedologie, Mendelova univerzita v Brně, Zemědělská 3, 613 00 Brno, Česká republika, e-mail: xdundek@node.mendelu.cz, ladisholik@seznam.cz, toro83@centrum.cz, xhromad4@node.mendelu.cz, vranova@mendelu.cz, kr@mendelu.cz., formanek@mendelu.cz