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Organic Acids, Sugars, and L-Tryptophane in Exudates of Vegetables Growing on Stonewool and Their Effects on Activities of Rhizosphere Bacteria

Faina Kamilova,[1] Lev V. Kravchenko,[2] Alexander I. Shaposhnikov,[2] Tatiyana Azarova,[2] Nataliya Makarova,[2] and Ben Lugtenberg[1]

[1]Leiden University, Institute of Biology, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands; [2]All Russian Institute of Agricultural Microbiology, 3 Podbelsky Shossee, Saint-Petersburg-Pushkin, Russia

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The influence of stonewool substrate on the exudation of the major soluble carbon nutrients and of the auxin precursor tryptophane for *Pseudomonas* biocontrol agents was studied. To this end, the composition of the organic acids and sugars, as well that of tryptophane, of axenically collected exudates of seed, seedlings, and roots of tomato, cucumber, and sweet pepper was determined. The major results were as follows. i) The total amount of organic acid is much higher than that of total sugar. ii) Exudation of both organic acids and sugars increases during plant growth. iii) Citric, succinic, and malic acids represent the major organic acids, whereas fructose and glucose are the major sugars. iv) Compared with glass beads as a neutral substrate, stonewool substantially stimulates exudation of organic acids and sugars. v) It appeared that enhanced root-tip-colonizing bacteria isolated previously from the rhizosphere of tomato and cucumber grow much better in minimal medium with citrate as the sole carbon source than other, randomly selected rhizobacteria do. This indicates that the procedure which selects for excellent root-tip colonizers enriches for strains which utilize the major exudate carbon source citrate. vi) The content of L-tryptophane, the direct precursor of auxin, is approximately 60-fold higher in seedling exudates of tomato and sweet pepper than in cucumber seedling exudates, indicating a higher possibility of plant growth stimulation after inoculation with auxin-producing rhizobacteria for tomato and sweet pepper crops than for cucumber. However, the biocontrol strain *Pseudomonas fluorescens* WCS365, which is able to convert tryptophane into auxin, did not stimulate growth of these three crops. In contrast, this strain did stimulate growth of roots of radish, a plant which exudes nine times more tryptophane than tomato does.

After introduction of bacterial strains into the rhizosphere (e.g., for biocontrol purposes), their colonization of the host root is mediated to a major extent by root exometabolites (Aliken and Smucker 1996; Hiltner 1904; Kuiper et al. 2002; Lugtenberg et al. 2001). The composition of exometabolites secreted by germinating seed and roots is dependent on plant species, plant age, root zone, plant ploidy, the plant's mineral nutrition, stress effects (Baudoin et al. 2002; Griffiths et al. 1999; Hodge et al. 1998; Kravchenko et al. 1993; Maloney et al. 1997; Vancura 1964, 1967; Yang and Crowly 2000), and other environmental factors such as the presence of microbes (Meharg and Kilham 1995; Prikryl and Vancura 1980) and the secondary metabolite 2,4 diacetyl phloroglucinol (Phillips et al. 2004). During growth of new roots, exudate secreted by the zone of elongation and by the root apex supports mainly those microbes which use the rapidly utilizable organic acids and sugars. Exudate compounds other then sugars and amino acids have received less attention, though organic acids play an important role in cell metabolism, effect activity of microflora, and are good metal-chelating agents (Curl and Truelove 1986). In the old root zones, where the rhizodeposits mainly consist of plant cells and mainly contain hardly utilizable substrates, those fungi and bacteria dominate which are adapted to oligotrophic growth conditions (Folman et al. 2001).

Deficiency of P and Fe (Dakora and Phillips 2002; Hoffland et al. 1989, 1992; Johnson et al. 1996) or elevated concentrations of Al^{3+} ions lead to increasing exudation of organic acid anions in some species (Li et al. 2002, Pirenos et al. 2002).

Dicarboxylic acids are potential chelating agents for positively charged metal ions such as Al^{3+}. They may play an important role in protecting the plant against aluminum incorporation (Ahonen-Jonnarth et al. 2000; Christiansen-Weniger et al. 1992, Ma et al. 1997).

It was demonstrated previously that the organic acid fraction represents the major class of utilizable carbon of tomato root exudate (de Weert et al. in press; Lugtenberg et al. 2001). Organic acid utilization by rhizobacteria is crucial for competitive tomato root tip colonization, a process which is often essential for biocontrol (Chin-A-Woeng et al. 2000, Kamilova et al. 2005). Utilization of sugars plays a minor role, if any (Lugtenberg et al. 1999). Plant roots secrete such low amounts of auxin that the auxin concentration in exudates is usually too low to determine. Many rhizobacteria are able to convert tryptophane from root exudates into auxin, which can result in enhanced growth of the plant (Frankenberger and Arshad 1995).

Tomato, cucumber, and sweet pepper are major horticultural crops. Traditionally, these vegetables are grown in soil; today, however, there is a strong shift to growth on stonewool substrate because growth can be better controlled, which results in a higher yield. Stonewool, a product of melted rock, is practically sterile at the beginning of plant growth. Therefore, the young plants are vulnerable to attack by pathogens. A rhizosphere microflora builds up in the course of plant growth and can become disease-suppressive (Postma et al. 2000). Plant diseases in stonewool can be controlled by microbes (Duffy and Defago 1997; Paulitz and Bélanger 2001; Postma et al. 2000). In order to understand biocontrol in stonewool, it is important to know the nature of the major utilizable carbon sources in exudates of horticultural

Corresponding author: F. Kamilova; E-mail: Kamilova@rulbim.leidenuniv.nl

crops growing on this substrate. An additional reason is that stonewool contains aluminum ions (Luoto et al. 1994) that may lead to a high organic acid content in root exudate, as explained previously. As controls, we used plants grown on glass beads.

Selection of the competitive tomato root tip colonizers via recently developed enrichment procedure results in the isolation of strains which grow highly efficiently on root exudates. Many of these enhanced root-tip colonizers appeared to be able to control tomato foot and root rot. Therefore, it is likely that these bacteria act through competition for nutrients and niches on the root (Kamilova et al. 2005).

The aims of the research described in this article are to i) determine the composition of organic acids and sugars of seed, seedling, and root exudates of tomato, cucumber, and sweet pepper grown on stonewool substrate; ii) compare the root exudate composition of plants grown on stonewool with control plants growing on glass beads; iii) test whether bacteria selected for enhanced root-tip colonization after bacterization of seed (Kamilova et al. 2005) were in fact selected for fast growth on the major exudate carbon source; iv) determine the potential of the biocontrol agent *Pseudomonas fluorescens* WCS365 to produce auxin in the rhizospheres of plants growing on stonewool by measuring the tryptophane levels in exudates; and v) determine whether the levels of auxin produced by this efficient colonizing are sufficiently high to stimulate plant growth.

RESULTS

Organic acids and sugars.

Tomato. The total amount of organic acids per seed and per plant (Table 1) increased with plant growth: seedling and root exudates contained 2 and 26 times more organic acids than seed exudate. Citric acid, malic acid, and succinic acid were the main organic acids in seed and seedling exudates. Citric acid was the major organic acid in all three stages of plant growth, whereas the percentage of malic acid drastically decreased and that of succinic acid strongly increased when seedlings became plants (Table 1).

The total amount of sugars per seed and per plant (Table 2) increased with plant growth: seedling and root exudates contained 2.7 and 5.3 times more sugars than seed exudate. Xylose, glucose, and fructose appeared to be the major sugars. Xylose was the main sugar in seed and seedling exudates, whereas fructose and glucose were dominant in root exudate (Table 2).

Exudates of seed and seedlings contained significantly more organic acids than sugars. Comparison of root exudates of plants grown on the inert substrate glass beads with those grown on stonewool revealed a clear positive effect of the latter substrate on the amount of exuded organic acids and sugars. On stonewool, tomato roots exuded five times more organic acids and sugars than on glass beads. On both substrates, citric and succinic acids were the major organic acids and fructose and glucose were the major sugars (compare Tables 1 and 2).

Cucumber. Analysis of the exudates of cucumber grown on stonewool showed that, for this crop also, the total amount of organic acids (Table 3) and sugars (Table 4) per seed and plant increased within plant growth. Citric acid was the major organic acid found in seed and root exudates whereas, surprisingly, succinic acid was dominant in seedling exudate (Table 3). Glucose and fructose were the major sugars. Fructose was the major sugar in seed exudate, whereas glucose was dominant in seedling and root exudates (Table 4). In all stages of plant growth, the amount of organic acids was higher than that of sugars; however, in root exudate, the difference was much less for cucumber (3.9-fold) than for tomato (10.2-fold). As was observed for tomato, growth of cucumber on stonewool resulted in a higher exudation of organic acids and especially of sugars than growth on glass beads. On both substrates citric, malic and succinic acids constituted the major organic acids. However, and in contrast to what was observed for tomato (Table 1), malic acid was the major compound (49.1%) on glass beads, whereas citric acid was the dominant acid (72.5%) on stonewool. Citric acid constituted 58% of the total pool of organic acids and sugars on stonewool. In root exudates of plants cultivated on glass beads, the major sugars fructose and glucose were present in approximately the same amount (40.8 and 44.2%, respectively), whereas maltose comprised approximately 12% of the total amount of sugar. Under stonewool conditions, the percentage of glucose increased at the expense of fructose and maltose (Table 4).

Sweet pepper. For this crop as well, the total amount of organic acids (Table 5) and sugars (Table 6) per seed and per plant increased with plant growth. The organic acid fraction (Table 5) was higher than the sugar fraction (Table 6) in all stages of plant growth. Citric acid, succinic acid, and malic acid were the major organic acids (Table 5). Succinic acid was dominant in seed exudate, whereas citric acid was the major organic acid in seedling and root exudates (Table 5). Fructose was the major sugar in all three exudates, followed by glucose, whereas a small but significant amount of maltose was present in all exudates (Table 6). In comparison with growth on glass beads, stonewool stimulated the exudation by the plant root of sugars especially and, to a minor extent, of organic acids also. On both substrates, citric acid was the major compound found and consisted of 40 and 32% of total organic acids and sugars, respectively. Growth on stone-

Table 1. Organic acid composition of tomato seed, seedling, and root exudate

	Amount of organic acid[a]					
	Acid, µg/plant ± SD (%)[b]				Acid, µg/mg of dry plant weight ± SD (%)[b]	
	Stonewool			Glass beads	Stonewool	Glass beads
Acid	Seed[c]	Seedling[d]	Root[e]	Root	Root[e]	
Citric	3.2 ± 0.7 (47.1)	6.2 ± 2.1 (46.9)	110.4 ± 2.4 (62.1)	26.0 ± 4.2 (70.2)	28.4 ± 2.1 (62.2)	6.8 ± 1.0 (69.9)
Piruvic	Trace	0.11 ± 0.1 (0.8)	ND	ND	ND	ND
Malic	2.7 ± 0.4 (39.8)	5.5 ± 1.2 (41.6)	2.3 ± 0.3 (1.3)	1.8 ± 0.5 (4.9)	0.6 ± 0.1 (1.3)	0.5 ± 0.1 (5.1)
t-Aconitic	ND	ND	0.06 ± 0.01 (0.03)	ND	0.03 ± 0.01 (0.1)	ND
Succinic	0.63 ± 0.11 (9.3)	0.82 ± 0.31 (6.2)	61.5 ± 2.7 (34.6)	9.1 ± 0.4 (24.5)	15.8 ± 0.4 (34.4)	2.4 ± 0.1 (24.6)
Fumaric	Trace	0.12 ± 0.05 (0.90)	0.30 ± 0.04 (0.2)	0.14 ± 0.04 (0.4)	0.09 ± 0.03 (0.2)	0.04 ± 0.01 (0.4)
Propionic	ND	Trace	ND	ND	ND	ND
Pyroglutamic	0.26 ± 0.12 (3.8)	0.48 ± 0.22 (3.6)	3.1 ± 0.2 (1.7)	ND	0.78 ± 0.06 (1.7)	ND
Total amount	6.79	13.23	177.66	37.04	45.60	9.74

[a] Results are the mean of three high-performance liquid chromatography analyses. SD = standard deviation and ND = not detected.
[b] Percentage of total organic acid.
[c] Seed exudate was collected after 2 days at 4ºC.
[d] Seedling exudate was collected after 4 days at 21ºC.
[e] Root exudate was collected after 14 days at 21ºC.

wool increased the percentage of succinic acid and decreased the fraction of malic acid.

Does enrichment for enhanced colonizing bacteria enrich for strains that grow well on the major exudates' carbon source?

In a previous article (Kamilova et al. 2005), we described the isolation of very competitive root-tip-colonizing bacteria, a substantial fraction of which appeared to be able to control tomato foot and root rot. Because these strains grow rapidly on tomato root exudate, we suggested that competition for nutrients contributes to their mechanism of biocontrol. Our present results indicate that citric acid is the major detected carbon source of stonewool-grown tomato; therefore, we wondered whether enhanced colonization also correlates with fast growth on citrate as the sole carbon source. Four of the excellent root-tip colonizers (Kamilova et al. 2005) were grown in BM medium (Lugtenberg et al. 1999) with 300 μM citrate as the sole carbon source, a concentration which approximately corresponds to the maximum concentration found in the root exudate of tomato plants grown on stonewool. As a control, we used five randomly selected rhizobacteria isolated from the

Table 2. Sugar composition of tomato seed, seedling, and root exudate growing on different substrates

	Amount of sugar[a]					
	Sugar, μg/plant ± SD (%)[b]				Sugar, μg/mg of dry plant weight ± SD (%)[b]	
	Stonewool			Glass beads	Rockwool	Glass beads
Sugar	Seed[c]	Seedling[d]	Root[e]	Root	Root[e]	
Glucose	0.93 ± 0.16 (28.5)	1.73 ± 0.25 (19.7)	5.85 ± 0.48 (33.7)	1.24 ± 0.08 (37.2)	1.51 ± 0.19 (33.8)	0.32 ± 0.03 (37.2)
Fructose	0.73 ± 0.13 (22.4)	2.60 ± 0.35 (29.6)	10.53 ± 0.75 (60.6)	1.91 ± 0.22 (57.4)	2.71 ± 0.30 (60.6)	0.50 ± 0.03 (58.1)
Maltose	0.12 ± 0.04 (3.7)	0.20 ± 0.06 (2.3)	0.36 ± 0.06 (2.1)	0.04 ± 0.02 (1.2)	0.09 ± 0.01 (2.0)	0.01 ± 0.01 (1.2)
Ribose	Trace	0.22 ± 0.08 (2.5)	0.54 ± 0.01 (3.1)	0.10 ± 0.01 (3.0)	0.14 ± 0.01 (3.1)	0.03 ± 0.01 (3.5)
Xylose	1.48 ± 0.32 (45.4)	4.02 ± 0. 91 (45.8)	0.09 ± 0.01 (0.5)	0.04 ± 0.002 (1.2)	0.02 ± 0.01 (0.5)	Trace
Total amount	3.26	8.77	17.37	3.33	4.47	0.86

[a] Results are the mean of three high-performance liquid chromatography analyses. SD = standard deviation.
[b] Percentage of total sugar.
[c] Seed exudate was collected after 2 days at 4ºC.
[d] Seedling exudate was collected after 4 days at 21ºC.
[e] Root exudate was collected after 14 days at 21ºC.

Table 3. Organic acid composition of cucumber seed, seedling, and root exudate

	Amount of organic acid[a]					
	Acid, μg/plant ± SD (%)[b]				Acid, μg/mg of dry plant weight ± SD (%)[b]	
	Stonewool			Glass beads	Stonewool	Glass beads
Acid	Seed[c]	Seedling[d]	Root[e]	Root	Root[e]	
Citric	17.9 ± 2.7 (78.9)	17.3 ± 2.8 (31.1)	93.4 ± 7.7 (72.5)	10.4 ± 4.5 (16.3)	2.78 ± 0.19 (72.4)	0.30 ± 0.13 (15.4)
Piruvic	0.30 ± 0.11 (1.3)	0.64 ± 0.18 (1.1)	ND	ND	ND	ND
Malic	0.51 ± 0.18 (2.2)	0.47 ± 0.11 (0.9)	17.8 ± 0.3 (13.8)	31.3 ± 3.7 (49.1)	0.53 ± 0.01 (13.8)	0.9 ± 0.1 (46.2)
t-Aconitic	0.04 ± 0.01 (0.2)	ND	0.03 ± 0.01 (0.02)	ND	Trace	ND
Succinic	2.9 ± 0.4 (12.8)	31.2 ± 5.3 (56.1)	14.9 ± 3.2 (11.6)	20.4 ± 3.0 (32.0)	0.44 ± 0.10 (11.4)	0.70 ± 0.17 (35.9)
Fumaric	0.15 ± 0.09 (0.6)	0.11 ± 0.09 (0.2)	0.85 ± 0.02 (0.6)	1.0 ± 0.1 (1.6)	0.03 ± 0.01 (0.8)	0.03 ± 0.01 (1.5)
Pyroglutamic	0.90 ± 0.12 (4.0)	5.9 ± 1.2 (10.6)	1.9 ± 0.1 (1.5)	0.7 ± 0.1 (1.0)	0.06 ± 0.02 (1.6)	0.02 ± 0.01 (1.0)
Total	22.7	55.62	128.9	63.80	3.84	1.95

[a] Results are the mean of three high-performance liquid chromatography analyses. SD = standard deviation and ND = not detected.
[b] Percentage of total organic acid.
[c] Seed exudate was collected after 2 days at 4ºC.
[d] Seedling exudate was collected after 4 days at 21ºC.
[e] Root exudate was collected after 14 days at 21ºC.

Table 4. Sugar composition of cucumber seed, seedling, and root exudate

	Amount of sugar[a]					
	Sugar, μg/plant ± SD (%)[b]				Sugar, μg/mg of dry plant weight ± SD (%)[b]	
	Stonewool			Glass beads	Stonewool	Glass beads
Sugar	Seed[c]	Seedling[d]	Root[e]	Root	Root[e]	
Glucose	2.6 ± 0.6 (32.2)	11.2 ± 2.9 (49.6)	19.3 ± 2.3 (58.3)	1.3 ± 1.0 (44.2)	0.57 ±0.06 (58.2)	0.04 ± 0.01 (45.3)
Fructose	4.4 ± 1.3 (54.5)	6.4 ± 1.1 (28.3)	12.17 ± 0.17 (36.7)	1.2 ± 0.6 (40,8)	0.36 ± 0.01 (36.7)	0.03 ± 0.01 (39.9)
Maltose	0.23 ± 0.05 (2.9)	1.1 ± 0.3 (4.9)	1.1 ± 0.2 (3.3)	0.35 ± 0.1 (11.9)	0.03 ± 0.01 (3.1)	0.01 ± 0.01 (11.9)
Melibiose	0.02 ± 0.01 (0.2)	Trace	ND	ND	ND	ND
Ribose	0.12 ± 0.08 (1.5)	1.2 ± 0.3 (5.3)	0.30 ± 0.04 (0.9)	ND	0.01 ± 0.01 (1.0)	ND
Xylose	0.70 ± 0.10 (8.7)	2.7 ± 0.7 (11.9)	0.26 ± 0.03 (0.8)	0.09 ± 0.02 (3.1)	0.01 ± 0.01 (1.0)	Trace
Total amount	8.07	22.6	33.13	2.94	0.98	0.08

[a] Results are the mean of three high-performance liquid chromatography analyses. SD = standard deviation and ND = not detected.
[b] Percentage of total sugar.
[c] Seed exudate was collected after 2 days at 4ºC.
[d] Seedling exudate was collected after 4 days at 21ºC.
[e] Root exudate was collected after 14 days at 21ºC.

sample before starting the enrichment procedure (Kamilova et al. 2005). The four enhanced root colonizers reached densities of 7×10^6 cells/ml after 24 h. In contrast, control bacteria grew poorly or did not grow at all (Fig. 1).

Tryptophane levels in exudates.

The levels of tryptophane in seedling exudate of tomato, cucumber, and sweet pepper grown on stonewool were determined and, as will be explained later, compared with that in exudate of radish grown on filter paper. The amount of tryptophane in the exudates of radish was the highest, both per seedling as well as per seed weight. The results obtained for plants grown on stonewool showed that the tryptophane level per seedling was lowest for cucumber and highest for tomato (Table 7). On the basis of seed weight, the amount of tryptophane was comparable for tomato and sweet pepper and was approximately 60 times higher than in cucumber exudate, but approximately eight to nine times lower than in radish exudate.

Effect of the biocontrol strain *P. fluorescens* WCS365 on plant biomass.

P. fluorescens strain WCS365, an excellent biocontrol strain of tomato foot and root rot, was tested for its ability to produce auxin in the presence of exogenous tryptophane.

After incubation for 8 days in King's medium B (KB) supplemented with tryptophane at 500 µg/ml, the strain appeared to produce 7 to 8 µg of auxin per milliliter of medium. When seed of tomato, cucumber, or sweet pepper were bacterized with this strain, no significant growth stimulation of the shoot fresh or dry weight in both soil and stonewool was observed. Because radish exudes extremely high amounts of tryptophane (Table 7), we tested the effect of bacterization on the root weight of this plant and found that bacterization significantly increased the dry weight of radish roots from 0.15 ± 0.07 to 0.26 ± 0.07 g/root.

DISCUSSION

The aim of the present research was to identify the major organic acids and sugars available for pathogens and biocontrol agents on the roots of the major crops grown on stonewool, namely tomato, cucumber, and sweet pepper. Information on the content and composition of organic acids and sugars in exudates of the mentioned crops is limited and has a very fragmented character (Rovira 1969; Vancura and Hovadik 1965). There is no data available on the effect of growth substrates on root exudate composition.

Our results show that, in all stages of tomato growth in stonewool, citric acid was the major organic acid in root exudates (Table 1). Malic acid and succinic acid also were important, but their levels were very dependent on the plant age. The percentage of malic acid strongly decreased between the seedling and root stage, whereas the opposite occurred with succinic acid, which became a major organic acid between these growth stages (Table 1). In the exudates of cucumber (Table 3) and sweet pepper (Table 5) as well, citric, malic, and succinic acids were present in substantial amounts, although the levels and timing could differ between the three crops.

Glucose, fructose, and xylose were the major sugars detected in the exudates of stonewool-grown tomato. Whereas glucose and fructose remained the major components in all growth stages of tomato, the percentage of xylose dropped dramati-

Table 5. Organic acid composition of sweet pepper seed and root exudate growing on different substrates

	Amount of organic acid[a]					
	Acid, µg/plant ± SD (%)[b]				Acid, µg/mg of dry plant weight ± SD (%)[b]	
	Stonewool			Glass beads	Stonewool	Glass beads
Acid	Seed[c]	Seedling[d]	Root[e]	Root	Root[e]	
Citric	0.36 ± 0.04 (15.3)	21.4 ± 0.7 (51.0)	32.8 ± 0.9 (51.5)	21.9 ± 4.5 (45.3)	5.4 ± 0.14 (51.5)	3.1 ± 0.51 (45.3)
Malic	0.68 ± 0.12 (28.8)	1.6 ± 0.3 (3.8)	5.0 ± 0.6 (7.9)	14.8 ± 2.9 (30.6)	0.82 ± 0.10 (7.9)	2.1 ± 0.3 (30.6)
Succinic	1.17 ± 0.02 (49.6)	15.8 ± 1.6 (37.6)	22.7 ± 3.1 (35.6)	11.3 ± 1.3 (23.5)	3.7 ± 0.11 (35.6)	1.6 ± 0.2 (23.5)
Fumaric	Trace	0.096 ± 0.003 (0.2)	0.23 ± 0.05 (0.3)	0.26 ± 0.03 (0.6)	0.04 ± 0.003 (0.3)	0.04 ± 0.002 (0.6)
Pyroglutamic	0.15 ± 0.02 (6.3)	3.1 ± 0.2 (7.4)	3.0 ± 0.4 (4.7)	ND	0.49 ± 0.05 (4.7)	ND
Total amount	2.36	42.0	63.7	48.3	10.5	6.84

[a] Results are the mean of three high-performance liquid chromatography analyses. SD = standard deviation and ND = not detected.
[b] Percentage of total organic acid.
[c] Seed exudate was collected after 2 days at 4ºC.
[d] Seedling exudate was collected after 14 days at 21ºC.
[e] Root exudate was collected after 21 days at 21ºC.

Table 6. Sugar composition of sweet pepper seed and root exudate growing on different substrates

	Amount of sugar[a]					
	Sugar, µg/plant ± SD (%)[b]				Sugar, µg/mg of dry plant weight ± SD (%)[b]	
	Stonewool			Glass beads	Stonewool	Glass beads
Sugar	Seed[c]	Seedling[d]	Root[e]	Root[e]	Root[e]	
Glucose	0.34 ± 0.10 (36.6)	4.60 ± 0.08 (35.4)	14.7 ± 2.0 (37.8)	3.4 ± 0.5 (52.6)	2.4 ± 0.2 (37.8)	0.48 ± 0.04 (52.5)
Fructose	0.47 ± 0.11 (50.5)	7.87 ± 2.52 (60.5)	23.1 ± 0.4 (59.4)	2.8 ± 0.6 (43.3)	3.8 ± 0.2 (59.4)	0.40 ± 0.04 (43.3)
Maltose	0.08 ± 0.008 (8.6)	0.39 ± 0.19 (3.0)	1.1 ± 0.2 (2.8)	0.11 ± 0.02 (1.7)	0.18 ± 0.03 (2.8)	0.02 ± 0.01 (1.7)
Ribose	ND	0.01 ± 0.006 (0.1)	ND	0.07 ± 0.02 (1.0)	ND	0.01 ± 0.01 (1.1)
Xylose	0.04 ± 0.03 (4.3)	0.13 ± 0.02 (1.0)	ND	0.09 ± 0.08 (1.4)	ND	0.01 ± 0.01 (1.4)
Total amount	0.93	13.00	38.9	6.47	6.38	0.92

[a] Results are the mean of three high-performance liquid chromatography analyses. SD = standard deviation and ND = not detected.
[b] Percentage of total sugar.
[c] Seed exudate was collected after 2 days at 4ºC.
[d] Seedling exudate was collected after 14 days at 21ºC.
[e] Root exudate was collected after 21 days at 21ºC.

cally in root exudate (Table 2). The same pattern for these three sugars also was found in cucumber (Table 3) and sweet pepper (Table 5).

We conclude that comparison of the compositions of organic acids and sugars in exudates of tomato, cucumber, and sweet pepper growing on stonewool indicates profound similarities: exudates of all three crops contained more organic acid than sugar and the same organic acids and sugars were present in large amounts. However, as mentioned previously, some remarkable differences also existed for which it is difficult to provide an explanation.

Comparison of the exudates of the described crops with that of grass (Kuiper et al. 2002) showed that, in grass exudates, citric, malic, and succinic acid were major organic acids and that fructose and glucose and, to a minor extent, xylose, were major sugars.

However, two major differences were observed: i) the total amounts of sugars and organic acids in grass exudates were practically the same; and ii) whereas arabinose was not detected in exudates of the three horticultural crops, it was a major sugar in grass exudate. It is tempting to speculate that these differences are due to differences between monocots and dicots.

Comparison of the effects of the substrates stonewool and glass beads on the root exudate composition showed that stonewool stimulated exudation of both organic acids and sugars in all three crops (Tables 1 to 6). The substrates hardly affected the levels of the individual major organic acids in tomato root exudate (Table 1). In cucumber root exudate (Table 3) of stonewool-grown plants, citric acid was present in a much higher percentage than in root exudate of glass beads-grown plants. In the latter case, the low amount of citric acid was compensated for by higher amounts of malic and succinic acids (Table 3). In sweet pepper root exudate, citric and succinic acid were major organic acids on both substrates (Table 5). No spectacular differences were observed in the composition of the individual sugars in exudates of plants grown on different substrates (Tables 2, 4, and 6).

It is difficult to explain the differences observed between the composition of root exudates of plants grown on the two substrates, stonewool and glass beads (Tables 1 to 6). If one compares the amounts per milligram of plant dry weight, it appears that all three plants grown on stonewool exuded more sugars as well as organic acids. Whereas, for tomato, the increase was similar for sugars (Table 2) and organic acids (Table 1), the increase of the sugar fraction (Tables 4 and 6) was considerably higher than that of the organic acid fraction (Tables 3 and 5) for the other two crops. It is known (Luoto et al. 1994) that stonewool contains Al^{3+} ions, which have been described to overexpress (Koyma et al. 1999) or derepress citrate synthase (Pirenos et al. 2002). This could explain the spectacular increase of citrate, especially in cucumber (Table 3). However, it does not explain the increase of the sugars glucose and fructose (Table 4) in cucumber exudate. It is clear that other factors, such as, for example, differences in the re-utilization of exudate compounds by the plant (Phillips et al. 2004), must play a role.

Recently isolated via enrichment, enhanced tomato and cucumber root-tip colonizers can grow efficiently on tomato root exudates and most of them appeared to control tomato foot and root rot. Evidence was provided that they do so using a new the mechanism for bacteria, namely competition for niches and nutrients (Kamilova et al. 2005). In the present article, we have shown (Fig. 1) that these strains, in fact, have been selected for enhanced growth not only on exudate but also on the major exudate carbon source, citric acid, in a medium with a pH value of 5.5, the pH of exudate. The enhanced colonizers reached an at least 10-fold higher number of CFU than five randomly selected rhizosphere bacteria did. This result strongly supports the notion (Kuiper et al. 2002) that growth to a high level on major exudates components is an important trait for rhizosphere competence.

The importance of organic acids as carbon sources for growth of bacteria in the rhizosphere was shown previously by De Weert and associates (in press), who demonstrated that mutants of the good colonizer *P. fluorescens* WCS365 with mutations in genes encoding malate/quinone oxidoreductase or cis-aconitate hydratase, enzymes of the tricarboxylic acid cycle, are poor competitive colonizers of the tomato root compared with the parental strain.

The seedling stage of a plant is most sensitive to plant growth stimulation by auxin. Most auxin in the rhizosphere is derived from tryptophane secreted by the root and converted to auxin by some rhizosphere bacteria. Exudates of the horticultural plants tomato, cucumber, and sweet pepper grown in stonewool differed considerably in the level of tryptophane that they contained (Ta-

Table 7. Amount of L-tryptophane in seedling exudates[a]

Plant	Amount of tryptophane (ng)	
	Per seedling	Per milligram of seed
Tomato	7.39 ± 0.83	3.69 ± 0.48
Cucumber	1.81 ± 0.28	0.057 ± 0.02
Sweet pepper	23.9 ± 0.83	3.4 ± 0.05
Radish	293 ± 35	32.2 ± 4.8

[a] Tomato and cucumber seedling exudates were collected after 4 days of growth on stonewool at 21ºC. Sweet pepper seedlings exudates were collected after 14 days growth on stonewool at 21ºC. This stage of sweet pepper growth is approximately the same as 4-day-old seedlings of tomato and cucumber. Radish seedlings exudates were collected after 4 days of growth on filter paper at 21ºC

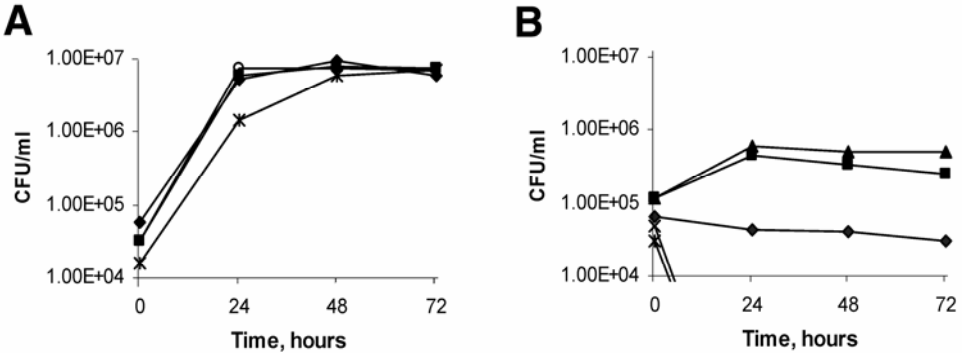

Fig. 1. Growth of rhizobacteria in BM medium (Lugtenberg et al. 1999) with 300 µM citrate (pH5.5) as the sole carbon source. **A,** Growth of enhanced tomato root-tip colonizers, isolated after enrichment for enhanced root colonizers (Kamilova et al. 2005). Tested enhanced colonizers were *Pseudomonas fluorescens* PCL1751 (stars), *P. fluorescens* PCL1753 (circles), *Pantoae agglomerans* PCA0067 (diamonds), and *Aeromonas hydrophila* PCA0081 (squares). **B,** Growth of five randomly chosen rhizobacteria isolated from rhizosphere of tomato and cucumber plants prior to applying the enrichment procedure.

ble 7). We tested whether inoculation of the seed of these plants with the auxin-producing bacterium *P. fluorescens* WCS365 resulted in growth stimulation of the shoot in both stonewool and soil. No significant growth stimulation was detected, presumably due to too-low levels of produced auxin. Therefore, we tested whether *P. fluorescens* WCS365 was able to stimulate growth of roots of radish, a plant which exudes much higher levels of tryptophane than the three studied horticultural crops (Table 7). Indeed, strain WCS365 appeared to be able to stimulate root growth of radish considerably. These results suggest that tryptophane levels in the rhizosphere of tomato, cucumber, and sweet pepper are insufficient to be stimulated in their growth by auxin-producing *P. fluorescens* WCS365, though one cannot exclude other factors, such as insufficient response of these crops to auxin or the presence of inhibitory substances for the auxin biosynthetic pathways in the tested bacterium.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

P. fluorescens WCS365 can control tomato foot and root rot (Dekkers et al. 2000; Kamilova et al. 2005) caused by *Fusarium oxysporum* f. sp. *radicis lycopersici.* It does so by inducing systemic resistance (Kamilova et al. 2005). To test its ability to produce auxin or to stimulate plant growth, bacteria were pregrown overnight in KB (King et al. 1954). In tests for auxin production, the medium was supplemented with tryptophane at 500 µg/ml.

Enhanced colonizing rhizobacteria were isolated as described by Kamilova and associates (2005). Briefly, rhizobacteria of cucumber and tomato plants were used to bacterize sterile seed, which subsequently were grown in a plant nutrient solution/quartz sand gnotobiotis system. After the root reached a length of approximately 10 cm, the root tip was removed; the bacteria were isolated and used for another enrichment for enhanced root-tip-colonizing bacteria. After a total of three cycles, the root-tip colonizers were tested for competitive root-tip colonizing ability against the good colonizer *P. fluorescens* WCS365. The following strains were equal or better colonizers than strain WCS365: *P. fluorescens* PCL1751, *P. fluorescens* PCL1753, *Pantoae agglomerans* PCA0067, and *Aeromonas hydrophila* PCA0081. These bacteria, as well as some rhizobacteria isolated from the same plants but not subjected to this enrichment procedure, were tested for growth on BM medium (Lugtenberg et al. 1999) with citric acid (300 µM) as the only carbon source. The cells were inoculated to a final concentration of 10^4 CFU/ml. The suspension was incubated at 21ºC under aeration at 150 rpm. Growth was measured by dilution plating on KB.

Preparation of sterile exudates.

Exudates were prepared from the plant species tomato (*Lycopersicon esculentum* L.) cv. Carmello, cucumber (*Cucumis sativus L.*) cv. Grendel, and sweet pepper (*Capsicum annum* L.) cv. Spitfire. The same sample was used for the analyses of organic acids and of sugars. Therefore, the method of sample preparation was identical for organic acid and sugar analysis. All samples were prepared in triplicate. Seed and seedling exudates were prepared under sterile conditions as follows. For each assay, 150 seed of tomato and sweet pepper and 100 seed of cucumber were washed in running tap water during 2 h and incubated in 100 ml of 5% HClO solution for 3 min. Subsequently, seed were carefully washed with 1 liter of sterile water and allowed to swell for 2 days at 4ºC. Seedlings were allowed to germinate for 4 days at 21ºC in stonewool (Delta Grodan, Brinkman Agro B.V., 's-Gravenzande, The Netherlands) saturated in twofold diluted sterile nutrient solution in petri dishes in the dark. Nutrient solution contained $Ca(NO_3)_2$ $4H_2O$, 972 mg/liter; $MgSO_4$ $7H_2O$, 616 mg/liter; $NaNO_3$, 140 mg/liter; KH_2PO_4, 170 mg/liter; NH_4NO_3, 55 mg/liter; Fe-EDTA, 20 mg/liter; $ZnSO_4$ $7H_2O$, 1.7 mg/liter; $B_4Na_2O_7$ $7H_2O$, 3.35 mg/liter; $CuSO_4$ $5H_2O$, 0.25 mg/liter; and Na_2MoO_4 $2H_2O$, 0.12 mg/liter. After incubation for the indicated period of time, seed and seedling exudates were isolated from stonewool by three extractions with 100 ml of water each. Extracts were evaporated to dryness at 45ºC under vacuum, dissolved in 5 ml of water, and sterilized by membrane filtration (0.45-µm pore size).

Plants (50 for tomato and sweet pepper and 25 for cucumber) were cultivated under sterile conditions in 500-ml glass beakers containing a 2-cm-thick layer of glass beads or stonewool on the bottom saturated with nutrient solution (50 ml for tomato and cucumber plants; 70 ml for sweet pepper) for 14 and 21 days. In all, 30 tomato plants, 5 cucumber plants, or 10 sweet pepper plants were cultivated per flask. Per experiment, three flasks were used for tomato and sweet pepper and five flasks for cucumber. The nutrient solution was the same as for seed. The cultivation was carried out in a controlled plant growth chamber at 21ºC. During the 16-h day, a light intensity of 5,000 Lux was applied which was followed by 8-h dark intervals. For all three crops, seed exudates were collected after 2 days at 4ºC. In the case of tomato and cucumber plants, seedling exudates were collected after 4 days at 21ºC and root exudates were collected after 14 days at 21ºC. For sweet pepper, seedling exudate was collected after 14 days at 21ºC and root exudate was collected after 21 days at 21ºC.

Isolation, separation, and quantification of organic acids and sugars.

Quantitative analysis of organic acids was carried out using a JASCO LC-900 series high-performance liquid chromatography (HPLC) system (Jasco International Co., Ltd., Victoria, BC, Canada). Organic acids were separated using an ion-exchange SUPELCOGEL C-610H, (Supelco Gland, Gland, Switzerland) column (30 cm by 7.8 mm). The mobile phase was 10.0 mM H_3PO_4 at a flow rate of 0.8 ml/min. Separation was carried out at 30ºC. The wavelength of UV detector was 210 nm. Sugars were separated using a stainless steel column (size, 25.0 × 4.6 mm) filled with SUPELCOSIL LC-NH2 (Supelco) with a particle size of 5 µm. Separation was carried out at 30ºC. The mobile phase was a gradient of acetonitril in water (84 to 77%). For detection of reducing sugars, the method of postcolumn labeling was used (Mopper and Degens 1972). The eluant flow rate was 0.8 ml/min and that of the tetrazolium reagent was 0.2 ml/min. The temperature of the water bath containing the reaction coil was 90ºC and the reaction time was approximately 1 min. Adsorption at a wavelength of 487 nm was detected using an absorption detector Jasco UV.

Assays of tryptophane and auxin.

For tryptophane determination, seedlings were cultivated at 21ºC for 4 days in petri dishes containing stonewool saturated with nutrient solution. Tryptophane was extracted from stonewool using distilled water. The samples were concentrated under vacuum at 45ºC and the dry precipitate was dissolved in 0.5 ml of water. The concentration of tryptophane in root exudate was determined by HPLC using a reverse-phase column LiChrosorb RP-18 and 13.7% acetonitril and 0.22% acetic acid as the eluent.

Auxin was determined spectrophotometrically (Gordon and Weber 1951) as described by Kamilova and associates (2005).

Plant growth promotion test in stonewool and soil.

Growth promotion of tomato, cucumber, and sweet pepper by *P. fluorescens* strain WCS365 was carried out in potting soil (Jonkind grond B.V., Aalsmeer, The Netherlands) and stonewool by measuring the fresh and dry weight of shoots as described by Kamilova and associates (2005). Briefly, seed were

coated with a suspension of bacteria at 10^8 CFU/ml in 1% (wt/vol) methylcellulose (Sigma-Aldrich, St. Louis) in water and dried under airflow. Seed were sown individually in small pots containing potting soil or in stonewool cubes (4 by 4 by 4 cm). Plants were grown for 3 weeks in a greenhouse at 21ºC, 70% relative humidity, and 16 h of daylight. For each crop, 48 plants were tested. Growth promotion of radish (Saxa Nova, Syngenta B.V., Enkhuizen, The Netherlands) was evaluated in the same way except that the plants were grown only in potting soil and the fresh and dry weight of the roots was measured.

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