Differing Organic Acid Exudation Pattern Explains Calcifuge and Acidifuge Behaviour of Plants

GERMUND TYLER* and LENA STRÖM

Department of Ecology, University of Lund, Ecology Building, S-223 62 Lund, Sweden

Received: 23 June 1994 Accepted: 24 August 1994

Many vascular plant species are unable to colonize calcareous sites. Thus, the floristic composition of adjacent limestone and acid silicate soils differs greatly. The inability of calcifuge plants to establish in limestone sites seems related to a low capacity of such plants to solubilize and absorb Fe or phosphate from these soils. Until now, mechanisms regulating this differing ability of plants to colonize limestone sites have not been elucidated. We propose that contrasting exudation of low-molecular organic acids is a major mechanism involved and show that germinating seeds and young seedlings of limestone plants exude considerably more di- and tricarboxylic acids than calcifuges, which mainly exude monocarboxylic acids. The tricarboxylic citric acid is a powerful extractor of Fe, and the dicarboxylic oxalic acid a very effective extractor of phosphate from limestone soils. Monocarboxylic acids are very weak in these respects. The study is based on ten species from limestone soils and ten species from acid silicate soils.

Key words: Calcifuge, calcicole, acidifuge, plant, organic acid, citrate, oxalate, exudation, germination.

INTRODUCTION

Calcifuge behaviour, or the inability of plants to establish, grow and compete successfully on limestone soils, is a well-recognized phenomenon and a classical field of ecological research. Calcifuge behaviour has often been explained by an inability of plants to solubilize adequate amounts of Fe from the soil or an inability to retain Fe as a metabolically active species in the tissues (Kinzel, 1982). Recently, several studies have shown that the forms of native phosphate of limestone soils are poorly available to many calcifuges, though cases of primary limitation by Fe have also been confirmed (Tyler, 1992, 1994; Tyler and Olsson, 1993). Germination is not affected but development beyond the seedling stage is not possible without soluble phosphate application, or (e.g., in *Galium saxatile* L.) repeated application of chelated Fe. The high pH (8.0–8.5) characteristic of the soil solution of limestone sites is, however, no problem *per se* to calcifuges we have tested.

Physiological mechanisms explaining the calcifuge behaviour of plants are not well understood. In a pilot study we exposed soil solution (pH 8.2), obtained by high-speed centrifugation of a limestone soil, to plant root exudates. Two calcifuges (*Rumex acetosella* L. and *Viscaria vulgaris* Bernh.) and two limestone plants (*Melica ciliata* L. and *Globularia vulgaris* L.) were treated and after 24 h the solutions were perfused through new subsamples of the same limestone soil. Solution which had been exposed to limestone plants solubilized measurable ($P < 0.01$; $n = 10$) quantities of phosphate and Fe from the soil, whereas solutions exposed to the calcifuges did not. From preliminary tests on concentrations of low-molecular organic

acids (LOAs) exuded by these plants we hypothesized that differences in the LOA exudation pattern might be responsible for the differences observed in Fe and phosphate solubilizing capacity.

This study examines the hypothesis that inherent differences in the amounts and proportions of organic acids exuded at seed germination and early seedling development may be important in controlling the calcifuge/acidifuge habit.

MATERIALS AND METHODS

This study is based on ten species inhabiting limestone soils (pH approx. 8) and ten species inhabiting acid silicate soils (pH 4–5; see Table 2 for species used).

Seeds were incubated aerobically in darkness, at 20 °C on continuously moist, H₂O-washed filter paper discs (diameter 90 mm) in transparent Petri dishes; five replicates of 0.25 g of each species. In a pilot study using an identical technique germination percentage had previously been controlled to vary 40–60% within 14 d. A few other species with lower germination percentage were not included. Ten blanks were treated in a similar way to the seed samples. After 10–14 d (depending on germination rate) 15 ml H₂O was added and 12 ml extracted after 5 min using a syringe, and deep frozen (–20 °C). Incubation continued for another 5 d, now with illumination (70 W m^{–2}) to facilitate seedling development and the same procedure repeated. The two sample solutions thus obtained from each dish were analysed and their sum is reported. LOA concentrations of the first solution were usually higher than in the second solution.

LOA concentrations of the liquids were determined by ion chromatography. The column system (Dionex) was composed of an anion trap column (ATC1, 4 mm, P/N 037151) to clean the eluent before sample injection, a guard

* For correspondence.

TABLE 1. Amounts of phosphate (P) and Fe (nmol g⁻¹ dry soil) extracted by eight LOAs from a limestone soil

	P	Fe
Lactic acid	2.3 ± 0.3	8.3 ± 1.2
Acetic acid	4.2 ± 0.5	9.9 ± 1.0
Formic acid	4.7 ± 0.5	7.1 ± 1.0
Malic acid	0.4 ± 0.1	29.9 ± 1.2
Succinic acid	5.4 ± 0.7	10.6 ± 1.0
Tartaric acid	2.4 ± 0.5	48.2 ± 2.2
Oxalic acid	17.9 ± 2.5	14.0 ± 1.0
Citric acid	0.8 ± 0.2	398.0 ± 5.9

Means of ten replicates with standard errors.

column (AG 11, 4 mm, P/N 044078), an analytical column (AS 11, 4 mm, P/N 044076) and an anion micro-membrane suppressor column (AMMS-II, 4 mm P/N 43074) inserted before the Conductomonitor III conductivity detector. A gradient of vacuum-degassed NaOH, 0.5 mM for 2.5 min, 0.5–5.0 mM for 3.5 min and 5.0–38.2 mM for 12 min, was used as the eluent; eluent flow rate being set at 2.0 ml min⁻¹. Injection volume was 100 µl. An autosampler was inserted in a cooling system to keep temperature at 6 °C. Samples and blanks were thawed immediately prior to analysis and filtered through an Acrodisc PF 0.8/0.2 µm ultrafilter. Organic acid anions determined were lactate, acetate, formate, malate + succinate, oxalate, citrate, isocitrate, cis-aconitate and trans-aconitate. Traces of other LOAs, mainly pyruvate and tartrate, were detected in some samples. Low concentrations (means and standard deviations) of formate, 2.0 (s.d. 0.8), lactate, 1.3 (s.d. 0.2), acetate, 0.9 (s.d. 0.2), and

oxalate, 0.6 (s.d. 0.1) nmol ml⁻¹, were determined in blanks (n = 10) and concentrations measured in sample liquids were reduced by these values. Blanks of other LOAs were below 0.1 nmol ml⁻¹.

To assess the capacity of different LOAs to solubilize Fe and phosphate from an Ordovician limestone soil, moist soil equivalent to 5 g dry weight was extracted for 1 h with 50 ml of 10 mM acid followed by centrifugation for 30 min. The ultrafiltered supernatant liquid (25 ml) was evaporated to dryness, treated with conc. HNO₃ and evaporated to 5 ml. Phosphate was analysed spectrophotometrically by the Mo-blue method and Fe by flame atomic absorption spectrophotometry.

RESULTS AND DISCUSSION

The solubilizing power on Fe and phosphate in a limestone soil of eight LOAs known to occur in exudates of cultivated plants (Vancura, 1964; Kovacs, 1971; Ohwaki and Hirata, 1992) was measured. The tricarboxylic citric acid was, by far the most powerful extractant of Fe, followed by the dicarboxylic tartaric, malic, succinic and oxalic acids, whereas the Fe solubilizing power of the monocarboxylic (formic, acetic, lactic) acids was very low. Oxalic acid proved to be the most potent solubilizer of limestone soil phosphate (Table 1).

The calcifuge and the acidifuge plants which were tested differed widely in their LOA exudation patterns and in the amounts released during germination and early seedling development (Table 2). Species of the limestone soils exuded three to four times more LOAs per unit of seed weight than species of the acid soils (Fig. 1).

TABLE 2. Low-molecular organic acid anions, µmol g⁻¹ seed, exuded from germinating seeds/seedlings of species from limestone soils and acid silicate soils

	Formate	Acetate	Lactate	Succinate + malate	Oxalate	Citrate	Isocitrate	Aconitate
Species of limestone soils								
<i>Melica ciliata</i>	0.20 (0.07)	0.01	0.01	2.52 (0.12)	0.31 (0.06)	0.25 (0.02)	0.08 (0.01)	0.19 (0.03)
<i>Artemisia campestris</i>	0.10 (0.04)	0.15 (0.04)	< 0.01	2.54 (0.16)	1.49 (0.14)	0.40 (0.08)	0.13 (0.03)	0.78 (0.06)
<i>Veronica spicata</i>	0.11 (0.04)	0.08 (0.03)	0.04 (0.01)	0.66 (0.06)	0.17 (0.02)	0.73 (0.04)	0.36 (0.08)	0.99 (0.09)
<i>Gypsophila fastigiata</i>	0.07 (0.02)	0.02 (0.01)	0.05 (0.01)	0.26 (0.02)	0.12 (0.02)	0.08 (0.02)	0.01	0.01
<i>Geranium sanguineum</i>	< 0.01	< 0.01	< 0.01	0.42 (0.08)	0.31 (0.08)	0.06 (0.01)	0.01	0.04 (0.01)
<i>Trifolium montanum</i>	0.23 (0.05)	0.01	0.01	0.60 (0.10)	0.41 (0.09)	0.13 (0.02)	0.14 (0.01)	0.06 (0.01)
<i>Helianthemum nummularium</i>	0.29 (0.06)	0.08 (0.03)	0.04 (0.02)	1.80 (0.14)	0.76 (0.09)	0.31 (0.09)	0.13 (0.04)	0.15 (0.06)
<i>Filipendula vulgaris</i>	0.15 (0.03)	0.24 (0.07)	0.14 (0.04)	1.86 (0.20)	0.76 (0.08)	0.30 (0.07)	0.07 (0.01)	0.05 (0.01)
<i>Galium triandrum</i>	0.24 (0.07)	0.20 (0.06)	0.18 (0.05)	1.60 (0.14)	0.39 (0.11)	0.95 (0.22)	0.32 (0.03)	1.56 (0.25)
<i>Oxytropis campestris</i>	0.10 (0.03)	0.02 (0.00)	0.02 (0.01)	0.34 (0.04)	0.26 (0.02)	0.19 (0.02)	0.04 (0.01)	0.03 (0.01)
Species of silicate soils								
<i>Achillea millefolium</i>	0.01	0.01	0.08 (0.03)	0.06 (0.02)	0.01	0.01	< 0.01	< 0.01
<i>Hypericum perforatum</i>	0.06 (0.02)	0.09 (0.02)	0.10 (0.02)	0.44 (0.08)	0.04 (0.01)	0.03 (0.00)	< 0.01	< 0.01
<i>Agrostis vinealis</i>	0.60 (0.12)	0.16 (0.05)	0.12 (0.02)	0.62 (0.04)	0.24 (0.03)	0.02 (0.01)	0.01	0.24 (0.04)
<i>Viscaria vulgaris</i>	0.12 (0.03)	0.16 (0.04)	0.17 (0.04)	0.48 (0.03)	0.17 (0.04)	0.02 (0.00)	< 0.01	< 0.01
<i>Dianthus deltoideus</i>	0.05 (0.01)	0.08 (0.02)	0.07 (0.03)	0.26 (0.02)	0.02 (0.00)	0.04 (0.01)	0.03 (0.01)	< 0.01
<i>Deschampsia flexuosa</i>	0.46 (0.08)	0.09 (0.03)	0.14 (0.04)	0.78 (0.04)	0.18 (0.01)	0.05 (0.01)	0.03 (0.00)	0.01
<i>Jasione montana</i>	0.01	0.05 (0.01)	0.16 (0.07)	0.20 (0.01)	0.02 (0.00)	0.05 (0.01)	0.02 (0.00)	< 0.01
<i>Rumex acetosella</i>	0.21 (0.05)	0.07 (0.01)	0.09 (0.01)	0.16 (0.02)	0.21 (0.02)	0.05 (0.01)	0.01	0.03 (0.01)
<i>Silene nutans</i>	0.01	0.08 (0.04)	0.20 (0.07)	0.02 (0.01)	0.05 (0.01)	< 0.01	< 0.01	< 0.01
<i>Poa nemoralis</i>	0.13 (0.06)	0.05 (0.02)	0.47 (0.03)	0.34 (0.04)	0.05 (0.02)	0.05 (0.02)	< 0.01	0.10 (0.03)

Means of five replicates with standard errors.

FIG. 1. Mean amounts ($\mu\text{mol g}^{-1}$ seed) of LOA anions exuded from species of limestone ($n = 10$) soils (□) and acid silicate ($n = 10$) soils (▨), and significance (P level) of the differences between means. Number of replicates of each species = 5.

Even more pronounced were the differences among the two plant categories in the proportions of different LOAs in their exudates. The exudation of tricarboxylic anions, including citrate, was on average one order of magnitude greater for limestone species than for acid soil species. Exudation of dicarboxylic acids by limestone species was four to five times that from acid soil species. Exudation of the monocarboxylic formate and acetate did not differ between the two categories but approximately three times

FIG. 2. Relative molar share of mono-, di- and tricarboxylic acid anions in exudates of germinating seeds and young seedlings. Species of limestone soils: 1, *Melica ciliata* L.; 2, *Artemisia campestris* L.; 3, *Veronica spicata* L.; 4, *Gypsophila fastigiata* L.; 5, *Geranium sanguineum* L.; 6, *Trifolium montanum* L.; 7, *Helianthemum nummularium* (L.) Mill.; 8, *Filipendula vulgaris* Moench; 9, *Galium triandrum* Hyl.; 10, *Oxytropis campestris* L. Species of acid silicate soils: 11, *Achillea millefolium*; 12, *Hypericum perforatum* L.; 13, *Agrostis vinealis* Schreb.; 14, *Viscaria vulgaris* Bernh.; 15, *Dianthus deltoides* L.; 16, *Deschampsia flexuosa* (L.) Trin.; 17, *Jasione montana* L.; 18, *Rumex acetosella* L.; 19, *Silene nutans* L.; 20, *Poa nemoralis* L.

more lactate was exuded from the acid soil species than from the limestone species.

Comparing the relative proportions of LOAs exuded thus reveals clearcut differences in the exudation with no overlap between the patterns of the two plant categories (Fig. 2). It is interesting to note that even the exudate of the calcifuge *Rumex acetosella*, which has considerable amounts of oxalic acid in its leaf tissues, contained only moderate quantities of oxalate. Particularly high concentrations of oxalate were measured in exudates of the limestone species *Artemisia campestris* L., and of citrate in exudates of the limestone species *Galium triandrum* Hyl. and *Veronica spicata* L.

Much malate and/or succinate (not separable by the IC method used) was measured in the exudates of the

TABLE 3. Number of seedlings produced per gram of seed

Species of limestone soils		Species of silicate soils	
<i>Melica ciliata</i>	128 ± 4	<i>Achillea millefolium</i>	1500 ± 40
<i>Artemisia campestris</i>	460 ± 16	<i>Hypericum perforatum</i>	220 ± 12
<i>Veronica spicata</i>	1480 ± 120	<i>Agrostis vinealis</i>	580 ± 20
<i>Gypsophila fastigiata</i>	216 ± 8	<i>Viscaria vulgaris</i>	2160 ± 70
<i>Geranium sanguineum</i>	56 ± 4	<i>Dianthus deltoides</i>	3040 ± 60
<i>Trifolium montanum</i>	310 ± 16	<i>Deschampsia flexuosa</i>	650 ± 20
<i>Helianthemum nummularium</i>	136 ± 4	<i>Jasione montana</i>	8000 ± 160
<i>Filipendula vulgaris</i>	350 ± 18	<i>Rumex acetosella</i>	210 ± 8
<i>Galium triandrum</i>	76 ± 5	<i>Silene nutans</i>	590 ± 24
<i>Oxytropis campestris</i>	56 ± 6	<i>Poa nemoralis</i>	780 ± 30

Means of five replicates ± standard errors.

germinating plants, particularly in the limestone species (Fig. 1 and Table 2). Both malic and succinic acid are rather weak solubilizers of Fe and phosphate compared to citric and oxalic acid, respectively (Table 1). There was a close relationship ($r = 0.96$) between the amounts of citrate and isocitrate exuded. Although not tested directly, isocitric and citric acid may have similar Fe solubilizing properties judging from their closely related chemical structure.

Sixteen of the species tested were herbaceous plants, whereas four were grasses. These grasses did not reveal any aberrations from the general edaphical pattern, except that they were all comparatively high in formate. The citrate and oxalate exudation of the limestone grass *Melica ciliata* was thus much higher than of the grasses of the acid soils.

The exudates of some species of limestone soils, *Galium triandrum*, *Veronica spicata* and *Artemisia campestris*, were comparatively high in the tricarboxylic aconitate. The solubilizing capacity of aconitate/aconitic acid has not been tested and no information on this question has been found in the literature.

In communicating the absolute amounts of LOAs exuded we preferred to use seed weight as the basis of calculation. Whereas germination percentage varied only within narrow limits (40–60%), mean weight per seed differed greatly among species as did the number of seedlings produced per vessel or unit of seed weight (Table 3). Species with small seeds give small seedlings which, on germination, might be supposed to produce less exudates per individual than species with large seeds/seedlings. As, on average, seeds and seedlings of the limestone soil species were larger than those of the silicate soil species, a calculation of LOA exudation per individual would have increased the absolute differences observed between species of the two plant categories. Some exudation of LOAs might also be expected from seeds

which do not successfully germinate. For these reasons we considered the unit of seed weight as the most adequate mode of calculating the absolute amounts exuded. And the most important finding, the great differences in the proportions of LOAs exuded, are not influenced by the mode of calculation.

We conclude that consistent differences exist between germinating plants of acid and limestone soils in respect of their exudation of low-molecular organic acids. Acidifuge plants of limestone soils exuded acids such as oxalate and citrate, which readily solubilize soil Fe and phosphate. The low exudation rates of oxalate and citrate from germinating seeds and young seedlings of plants adapted to acid soils may inhibit their establishment on limestone soils, where the pools of easily available Fe and phosphate are naturally very low.

LITERATURE CITED

- Kinzel H. 1982. *Pflanzenökologie und Mineralstoffwechsel*. Stuttgart: Eugen Ulmer.
- Kovacs MF. 1971. Identification of aliphatic and aromatic acids in root and seed exudates of peas, cotton, and barley. *Plant and Soil* 34: 441–451.
- Ohwaki Y, Hirata H. 1992. Differences in carboxylic acid exudation among phosphorus-starved leguminous crops in relation to carboxylic acid contents in plant tissues and phospholipid level in roots. *Soil Science and Plant Nutrition* 38: 235–243.
- Tyler G. 1992. Inability to solubilize phosphate in limestone soils—key factor controlling calcifuge habit of plants. *Plant and Soil* 145: 65–70.
- Tyler G. 1994. A new approach to understanding the calcifuge habit of plants. *Annals of Botany* 73: 327–330.
- Tyler G, Olsson PA. 1993. The calcifuge behaviour of *Viscaria vulgaris*. *Journal of Vegetation Science* 4: 29–36.
- Vancura V. 1964. Root exudates of plants. I. Analysis of root exudates of barley and wheat in their initial phases of growth. *Plant and Soil* 21: 231–248.