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Allelochemical root-growth inhibitors in low-molecular-weight cress-seed exudate

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Running title: Root-growth-inhibiting allelochemicals in cress-seed exudate

SUMMARY

Background and aims. Cress seeds release allelochemicals that overstimulate the elongation of neighbouring (potentially competing) seedlings' hypocotyls and inhibit their root growth. The hypocotyl promoter is potassium, but the root inhibitor was unidentified; its nature is investigated here.

Methods. Low-molecular-weight cress-seed exudate (LCSE) from imbibed *Lepidium sativum* seeds was fractionated by phase partitioning, paper chromatography, high-voltage electrophoresis and gel-permeation chromatography (on Bio-Gel P-2). Fractions, compared with pure potassium salts, were bioassayed for effects on *Amaranthus caudatus* seedling growth in the dark for 4 days.

Key results. LCSE robustly promoted amaranth hypocotyl elongation and inhibited root growth. The hypocotyl inhibitor was non-volatile, hot-acid-stable, hydrophilic, and resistant to incineration — as expected for K^+. The root inhibitor(s) had similar properties but were organic (activity lost on incineration). The root inhibitor(s) remained in the aqueous phase (at pH 2.0, 6.5 and 9.0) when partitioned against butan-1-ol or toluene, and were thus hydrophilic. Activity was diminished after electrophoresis, but the remaining root-inhibitors were neutral. They became undetectable after paper chromatography; therefore, they probably comprised multiple compounds, which partially separated from each other during fractionation. On gel-permeation chromatography, the root inhibitor co-eluted with hexoses.

Conclusions. Cress-seed allelochemicals inhibiting root growth are different from the agent (K^+) that over-stimulates hypocotyl elongation, and probably comprise a mixture of small, non-volatile, hydrophilic, organic substances. Abundant components identified chromatographically and by electrophoresis in cress-seed exudate fitting this description include glucose, fructose, sucrose and galacturonic acid. However, none of these sugars co-chromatographed and co-electrophoresed with the root-inhibitory principle of LCSE, and none of them (in pure form at naturally occurring concentrations) inhibited root growth. We conclude that the root-inhibiting allelochemicals of cress seed exudate remain unidentified.

INTRODUCTION

Many plants are capable of adversely affecting the growth and development of neighbouring potential competitors — a phenomenon known as allelopathy (Molisch, 1937; Rice, 1984; Willis, 2007; He *et al.*, 2019) and attributed to 'allelochemicals'. Some allelochemicals inhibit germination; others permit germination but interfere with the growth and development of 'target' seedlings. Such interference may include inhibition of growth or stimulation of excessive growth — adverse effects that could lead to the discovery of novel herbicides. For example, Syngenta's herbicide 'Callisto' contains mesotrione, an artificial analogue of the natural allelochemical leptospermone (Cornes, 2005; Dayan *et al.*, 2011; Cordeau *et al.*, 2016).

Allelopathic plants create and release allelochemicals that hinder the growth of surrounding roots (Asaduzzaman *et al.*, 2016; Yang and Kong, 2017), sometimes as an active response to chemicals from the presence of surrounding roots (Kong *et al.*, 2018; Li *et al.*, 2020). Conversely, the 'target' plant may detect the presence of the allelopathic species and modify root placement, thus avoiding the allelochemicals. Utilising allelopathy has the potential to be a special tool for weed control and sustainable agriculture because it is natural and environmentally benign. Allelochemicals may be more biodegradable than conventional herbicides but may also have unfavourable impacts on species other than those targeted. This makes ecological studies necessary before widespread use (Khan and Khan, 2015).

Understanding allelochemicals is also important for an appreciation of the ecological factors influencing the success of different plants in diverse natural environments. Allelochemicals arise from various parts of the 'donor' plant, including root exudates (Curl and Truelove, 1986; Fan *et al.*, 1997; Hale *et al.*, 1978; Rovira, 1969; Uren, 2000), and root volatiles (Jassbi *et al.*, 2010), fallen leaves (Sahu & Devkota, 2013; Szwed *et al.*, 2020; Sarkar *et al.*, 2012), seeds (Hasegawa *et al.*, 1992; Higashinakasu *et al.*, 2004; Boydston *et al.*, 2011) and stems (Li *et al.*, 2016). As a classic example, black walnut (*Juglans nigra*), one of the best-known allelopathic plants, generates the extremely effective allelochemical juglone from fallen leaves (Lee and Campbell 1969).

In this paper, we explore bioactive substances exuded from cress seeds (*Lepidium sativum*) which are known to interfere with the growth and development of neighbouring, potentially competing seedlings such as those of amaranth (*Amaranthus caudatus*), lettuce (*Lactuca sativa*) etc. (Hasegawa *et al.*, 1992; Iqbal *et al.*, 2012). Amaranth was chosen as the target species in the present work because of the small size

of its seedlings, such that numerous replicates can easily be performed in small Petri dishes, and because amaranth has previously been shown in the above-cited literature to be sensitive to allelochemicals. Previous work has confirmed that many species including *Arabidopsis thaliana*, *Helianthus annuus*, *Celosia cristata* and *Avena sativa* are targeted by cress-seed exudate (Higashinakasu *et al.*, 2004).

Cress-seed exudate overstimulates the elongation of amaranth hypocotyls and restricts their growth in girth while simultaneously inhibiting root growth (Hasegawa *et al.*, 1992; Yamada *et al.*, 2007; Iqbal *et al.*, 2012) — effects that would be detrimental to amaranth seedlings as model 'competitors'. Originally, the main allelochemical present in cress seed(ling) exudate was suggested to be lepidimoic acid (4-deoxy-β-L-*threo*-hex-4-enopyranuronosyl-(1→2)-L-rhamnose; an unsaturated acidic disaccharide derived from the pectic domain rhamnogalacturonan-I) (Hasegawa *et al.*, 1992; Yamada *et al.*, 2007). Subsequent work challenged this idea (Iqbal *et al.*, 2016). One of the cress-seed allelochemicals (the one responsible for over-stimulation of hypocotyl elongation) was shown to be inorganic potassium, K^+ (Fry 2017), which does indeed promote hypocotyl elongation in etiolated seedlings (McIntyre and Boyer, 1984). However, the active principle responsible for root growth inhibition was shown to be neither lepidimoic acid nor K^+, but instead an as-yet unidentified organic material (separable from lepidimoic acid and destroyed by ashing; Fry, 2017). Our hypothesis was that the root-growth-inhibiting principle was one or more organic substances — most likely a mixture of components — which we have partially characterised in the present work.

MATERIALS AND METHODS

Materials

Volatile chromatography solvents and electrophoresis buffers, filter paper discs (47 mm; Whatman No. 1), chromatography paper (Whatman No. 3) and general laboratory chemicals were sourced as described earlier (Fry, 2017). Plastic-backed silica-gel thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany; https://www.merckgroup.com/en). Bio-Gel P-2 was from Bio-Rad (Hercules, California, USA; https://www.bio-rad.com/en-uk/contact-us). Seeds, from E. W. King & Co. (Kelvedon, Colchester, Essex, UK; https://www.kingsseeds.com), were *Amaranthus caudatus* (code AMA001) and *Lepidium sativum* (Cress Fine Curled; code CRE03).

Preparation of low-M_r cress-seed exudate (LCSE)

LCSE was prepared as described by Iqbal *et al.* (2016). Cress seeds (5 g dry weight) were placed in a dialysis sac with a total of 100 ml water (about 50 ml inside the sac and 50 ml outside) for 48 h at 4°C in the dark. The external solution (LCSE; ~50 ml; total dissolved solids ~1.6 mg/ml) was filtered through filter-paper and stored frozen. This was the concentration of LCSE used in all bioassay experiments unless otherwise stated.

The osmolality of LCSE was estimated at ~11 mOsmol kg^{-1} by use of a freezing-point depression osmometer (Micro-Digital Osmometer MOD200 Plus, Camlab, Cambridge, UK; Rosko et al., 2017); see Table S1.

Properties of the bioactive principle(s) of LCSE

Fifteen independent preparations of LCSE were subjected to various treatments (1 ml LCSE for each treatment): freezing/thawing; freeze-drying; incubating in the presence of 0.25 M formic acid at 20 or 120°C for 1 h; ashing in a glass tube held over a Bunsen flame for 3 min at approximately 400°C; and partitioning between 20 mM aqueous formic acid (pH 2.7) and ethyl acetate. Controls included identical formic acid/water/ethyl acetate mixtures with no LCSE. All samples (except those that were simply frozen/thawed) were dried in a SpeedVac, re-dried several times from 0.1 ml water, and finally redissolved in 1 ml water. Finally, each solution was applied to amaranth seeds (see 'bioassay').

Bioassay

All 1-ml samples of treated LCSE (or fractions derived therefrom by chromatography or electrophoresis) were applied to two 4.7-cm discs of Whatman No. 1 filter paper in a 5-cm plastic Petri dish, and 10 amaranth seeds (well spaced) were added. The lids were sealed with Parafilm and the dishes incubated in the dark at 25°C for 4 days. The seedlings were stained with aniline blue (Long *et al.*, 2008; Fry, 2017) and the hypocotyls (white) and roots (stained blue) were measured with a ruler. The above bioassay procedure was also used in experiments investigating the biological effects of potassium salts and chromatography and electrophoresis solvents.

Each bioassay was conducted in at least three replicate Petri dishes. Experiments were also replicated: the data in Fig. 1 came from three complete repeats of the experiment conducted in 2019, 2022 and 2023, with the work in each year comprising 20–23 independently set up dishes; the data in Fig. 2 were also from three separate years, each with 15–22 independent LCSE preparations; data in Fig. 3 comprised four independent experiments (shown) plus an equal number of repeat runs; data in Figs. 4 and 5 were from three and two years' work, respectively, each performed with 15–20 independent LCSE preparations; Fig. 6 shows one representative experiment and two additional repeats are shown in Fig. S4.

Chromatography and electrophoresis

Whatman No. 3 papers (46 × 57 cm sheets) were washed by irrigation (as if for paper chromatography by the descending method) in 5% formic acid for 2 d, then in water for a further 5 d. The washing removed traces of ionic substances present in the paper. For paper chromatography, 100 µl of a 20-fold concentrated preparation of LCSE was applied as a 4 × 1-cm streak to 57-cm-long sheets of acid-washed Whatman No. 3 paper and developed by the descending method in BAW (butan-1-ol/acetic acid/water, 12:3:5) for 20 h. The paper was dried and re-dried, and fractions were eluted, as above.

For HVPE, similar LCSE samples were applied to 57-cm-long sheets of acid-washed Whatman No. 3 paper (with the LCSE samples loaded about half way between anode and cathode), which was then wetted with a volatile buffer [pyridine/acetic acid/H_2O (33:1:300 v/v/v), pH 6.5] at 2.5 kV for 13 min. The buffer was then removed in a stream of air and the paper was re-dried, and repeatedly dipped through acetone/methanol (2:1) and re-dried; strips of the paper were then eluted with water, and the eluate was freeze-dried and re-dissolved in water.

Ionic marker compounds, and LCSE tracks that were not going to be bioassayed, were stained by rapid dipping through bromophenol blue (0.4 g/l in ethanol containing 0.4 ml/l collidine) and hung to dry for about 15 min, revealing anions (yellow) and cations (blue). After drying, the same papers were stained for sugars with $AgNO_3$ (Fry, 2000).

For gel-permeation chromatography, as described by Iqbal *et al.* (2016), a 5.5-ml sample of concentrated LCSE was applied to a 185-ml bed-volume column of Bio-Gel P-2 and eluted in deionised water. The fractions collected were bioassayed on amaranth seedlings and small samples were analysed by TLC.

TLC was performed in silica-gel plates run in butan-1-ol/aceric acid/water (4:1:1 by vol.) and sugars were stained with thymol/H_2SO_4 (Jork *et al.*, 1994).

Phase partitioning of LCSE active principles

To test the effect of pH on the hydrophobicity of LCSE active principles, we dried 1.33 ml of LCSE (solid content 20 mg/ml) as an 8 × 1 cm streak on to Whatman No. 3 paper. Replicate papers were then wetted with a volatile buffer (formic acid/acetic acid/H_2O, 1:4:45, pH 2.0; pyridine/acetic acid/H_2O, 33:1:300, pH 6.5; or dilute ammonia, pH 9.0) and either (a) dried in a stream of air, (b) shaken in 100 ml butan-1-ol for 30 min then dried, or (c) shaken in 100 ml toluene for 30 min then dried. All papers were then thoroughly dried and any LCSE that remained on the paper was eluted with water, repeatedly freeze-dried and redissolved in water, and finally redissolved in 6.65 ml of water. This solution, which would have a concentration of 4 mg/ml (w/v) LCSE if no solutes had been lost into the organic solvents or remained bound to the paper, was bioassayed on amaranth seedlings. Control seedlings received pure water or never-dried LCSE.

Statistics and graphing

Data analysis was performed with the computer software Statistix for the analysis of variance at a significance level of p=0.05, usually better. For graphical representation, data from MS Excel files were transferred to SigmaPlot v14.0 to produce the histograms presented here.

RESULTS

Confirming the existence and basic properties of root-growth inhibitor

Amaranth seeds were incubated in the presence of various numbers of cress seeds. The presence of cress seeds had no effect on amaranth seed germination, but had two opposing effects on seedling growth (Fig. 1). The elongation of the amaranth hypocotyls was strongly promoted. The hypocotyl-promoting principle, previously suggested to be lepidimoic acid (Hasegawa *et al.*, 1992) has more recently been shown to be potassium ions (Fry 2017). The largest number of cress seeds tested (12) slightly diminished the promotion of amaranth hypocotyl elongation, probably because the elongation-promoting

effect of K^+ was countered by other products excreted by the cress; however, this effect was not statistically significant ($p \approx 0.1$).

The elongation of the amaranth roots was strongly inhibited by the presence of cress (Fig. 1). The nature of the presumed root-inhibiting allelochemical remained unknown and was therefore investigated in the present paper.

A priori, an effect of neighbouring cress seed(ling)s could be due to competition with the amaranth seedlings for an essential resource such as O_2 (in a sealed Petri dish), or it could be due to the secretion of cress allelochemical(s). The latter interpretation was supported by experiments in which we collected low-molecular-weight cress-seed exudate (LCSE) and applied this to amaranth in the absence of live cress (Fig. 2). This experiment clearly demonstrated a strong promoting effect of LCSE on amaranth hypocotyl growth (previously shown to be due to the presence of K^+; Fry, 2017) and a strong inhibitory effect on amaranth root growth.

The measured osmolality of LCSE was ~10.9 mOsmol kg^{-1} (Table S1), equivalent to ~11 mM of total osmotically active molecules and ions. This compares closely to the measured total solute concentration of LCSE, 1.6 mg/ml, which, if due entirely to hexoses, would correspond to ~9 mM (contributing ~0.022 MPa to the osmotic pressure). To investigate the possibility that osmotic pressure was the cause of the observed root growth inhibition (by opposing water uptake for cell expansion), we tested the effect of 0–64 mM glucose or KCl on amaranth seedlings (measured osmolality 0–129 mOsmol kg^{-1}). Glucose in this concentration range had no appreciable effect on seedling growth (either roots or hypocotyls; Table S1). KCl and a mixture containing glucose and KCl, also had a negligible effect on root growth, but promoted hypocotyl elongation, as expected for these concentrations of K^+. Thus osmotic pressure is unlikely to be the cause of root growth inhibition effected by LCSE.

To explore some fundamental properties of the root growth inhibitor, we subjected LCSE to various treatments and then re-tested its effect on amaranth seedlings (Fig. 2). Neither the hypocotyl promoter nor the root inhibitor was lost after drying or after incubating with hot or cold formic acid. Hot formic acid would have hydrolysed any lepidimoic acid, which was thus not the active principle. Both the hypocotyl promoter and the root inhibitor were found to partition into water, not ethyl acetate, indicating that the active principle was hydrophilic. The major difference was that the root inhibitor was lost upon ashing in a Bunsen flame, indicating that it was organic, whereas the hypocotyl promoter activity was retained. Thus, the root inhibitor

comprises heat-stable, hydrophilic, combustible compound(s) (thus not K^+), whose bioactivity is retained under conditions which would hydrolyse disaccharides, including lepidimoic acid.

Partitioning of LCSE active principles into organic solvents

We investigated the hydrophobicity of the active principles of LCSE at various pH values which might affect their ionisation if carboxy or amino groups are present. LCSE solutions buffered at pH 2.0, 6.5 or 9.0, applied to chromatography paper, were shaken with butanol or toluene or kept in the absence of partitioning solvents. Neither the hypocotyl promoter nor the root inhibitor was lost from the damp paper at any pH (Fig. S1). There appeared to be partial loss of both active principles into butanol from pH 9 solution. The main conclusion is that both active principles are hydrophilic at all three pH values, and therefore unlikely to be lipophilic weak acids (e.g. abscisic acid) or lipophilic weak bases (e.g. sphingenine).

Tolerance of amaranth seedlings to residues left after drying commonly used solvents

A promising approach for analysis of the active principles of LCSE would be chromatography or electrophoresis followed by bioassay of the separated fractions. Preliminary work suggested that some solvents commonly used during chromatography and electrophoresis might inhibit amaranth seedling growth. We therefore tested several solvents likely to be used (Fig. S2). Compared with pure water, the residues obtained after drying of two volatile electrophoresis buffers (pH 2.0 and 6.5), the coolant used for electrophoresis at pH 6.5 (toluene), and the paper chromatography solvent BAW all had little or no effect on amaranth germination and seedling growth. However, the coolant used for electrophoresis at pH 2.0 (white spirit; 'turpentine substitute') completely blocked germination (Fig. S2). The toxic constituent of white spirit could not be removed by repeated drying from alternative solvents e.g. toluene, acetone, methanol (data not shown). Thus, electrophoresis at pH 2.0 was not compatible with bioassays and was not further explored in this work. However, electrophoresis at pH 6.5 and chromatography in BAW were suitable separatory methods.

Deleterious effects of certain salts on root growth

As expected, all tested K^+ salts (at 5 or 10 mM) were able to promote amaranth hypocotyl elongation; Na^+ salts did not have this effect (Fig. 3). We investigated whether salts might be root growth inhibitors as such an effect would complicate future attempts to analyse the natural allelochemical(s) present in LCSE. Indeed, many of the tested salts tended to inhibit root growth, especially 10 mM salts of organic anions (Fig. 3a–c); however, phosphate and the chlorides were least inhibitory. These observations raise the possibility that LCSE fractions eluted from paper chromatograms or electrophoretograms that had been run in the presence of acetic or formic acid might inhibit root growth owing to the acetate or formate trapped by naturally occurring cations (K^+ being the most abundant cation in LCSE). Thus, added acetic or formic acid might convert endogenous K^+ into an apparent 'root inhibitory principle' of no biological significance.

Hypocotyl growth was promoted by the acetate, formate and chloride salts of K^+, but less so by the trifluoroacetate salt; root growth was inhibited by all the K^+ salts tested except the chloride (Fig. 3c). Thus, after chromatography or electrophoresis of LCSE specimens, it would be important to remove any formate, acetate or trifluoroacetate carried over from the solvents and which might otherwise be trapped in the sample by the K^+ and other cations present in LCSE.

We tested whether the root inhibiting effect of potassium acetate might be alleviated if we dried the sample from an excess of an alternative volatile acid such as HCl, formic acid or trifluoroacetic acid (TFA), whose anions might be less toxic. After drying, such samples would become predominantly KCl, K.formate and K.TFA respectively. Indeed, KOAc whose acetate had been largely removed by drying from HCl or TFA were less inhibitory to root growth than equimolar potassium acetate (Fig. 3d). Formic acid was not useful in this way. Thus an excess of HCl could potentially be used to drive off any trapped acetate or formate from K^+-containing fractions eluted from chromatograms and electrophoretograms. TFA is less suitable in this capacity as it itself interferes with the stimulatory effect of K^+ on hypocotyl growth (Fig. 3c,d).

The above data suggest that, even though K^+ is not the principal root inhibitor, it may interfere in root bioassays by forming non-volatile, growth-inhibitory salts when insufficiently dried from experimental samples, e.g. eluates from chromatograms and electrophoretograms.

Electrophoresis of LCSE at pH 6.5

Given that the bioactive principles do not partition into toluene, and that the buffers and toluene used can be removed by thorough drying, we characterised the root-inhibiting principle of LCSE by high-voltage electrophoresis at pH 6.5. LCSE contained abundant neutral sugars (not stained by bromophenol blue but detected by sugar stains; results not shown) and several ions including K^+, GalA, phosphate, sulphate and an unidentified anion (Fig. 4a). Root inhibitory activity was observed in electrophoretogram zone 6, which is where neutral and slow-migrating cationic solutes run. As expected, the hypocotyl promoter ran in the region of K^+. The results are compatible with the root inhibitor possessing no net charge at pH 6.5, thus possibly a neutral sugar. Neutral sugars, of course, do not separate from each other on electrophoresis so cannot be identified by this method.

Paper chromatography of LCSE

To gain further insight into the nature of the active principle(s), we fractionated LCSE by paper chromatography in butanol/acetic acid/water (12:3:5) and bioassayed zones of the chromatograms after thorough removal of the solvents. The mobilities of various relevant solutes in this chromatography system are shown (Fig. S3): In Fig. S3a, the ionic components are stained with bromothymol blue (anions stain yellow, cations blue or violet–orange in the case of Zn^{2+} and Cu^{2+}); in Fig. S3b, the sugars are stained brownish with silver nitrate (some ions show as pale spots against the weakly stained cellulose of the paper). LCSE contained detectable K^+, lepidimoic acid, GalA, glucose, fructose, sulphate, phosphate and an unidentified fast-migrating anion.

On separate chromatograms, 100-µl streak-loadings of 15 independent LCSE samples were similarly run alongside marker mixtures and identical streaks of the same 15 LCSE samples. As shown in Fig. 5 for five examples of the 15, the markers and one set of LCSE loadings were stained with bromophenol blue, revealing the ions present (Fig. 5a), followed by silver nitrate, revealing the sugars (Fig. 5b). Fractions ('zones 1–10') from the unstained LCSE run were then bioassayed for effects on amaranth seedling growth (Fig. 5c,d). The hypocotyl growth promoter was found (zones 4 > 5) corresponding to K^+, as expected. No significant inhibition of amaranth root growth was observed in any zone. It is possible that two or more components are required for detectable root inhibition and that they were separated by the chromatography.

Gel-permeation chromatography of LCSE

Gel-permeation chromatography (GPC) on Bio-Gel P-2 separates compounds principally on the basis of size and within the range ~100–2000 kDa. The constituents of LCSE were resolved into soluble polysaccharides in fractions 16–24, then a series of oligo- and monosaccharides, some of which can be provisionally identified on the TLC (glucose + fructose, peaking in fractions 36–42; sucrose in 34–40; galacturonic acid in 36–42; and a putative trisaccharide in 28–36) (Fig. 6). Indeed, trisaccharides such as raffinose have recently been reported to be present in LCSE (Lijina *et al.*, 2023). In addition, an unidentified fast-migrating sugar was observed in Bio-Gel fractions 48–50, previously referred to as B1 and shown to be a non-reducing, acidic disaccharide (Fig. 5 of Iqbal *et al.*, 2016). The peak of root inhibition ($p < 0.001$) was in fraction 38, coinciding with the monosaccharides; several neighbouring fractions also tended to inhibit root growth, though without statistical significance. The strong peak of hypocotyl promotion in fractions 30–38 was previously reported to be due to K^+ (Fry, 2017). Two further repeats of this experiment on a smaller scale confirmed all the above trends, with strong statistical significance (Fig. S4). The results indicate that root growth inhibition was attributable to small, water-soluble solute(s), of approximately the size of monosaccharides and apparently smaller than disaccharides (which would include lepidimoic acid).

DISCUSSION

Considerable interest centres on allelochemicals from the points of view of basic plant physiology, applications in agriculture including 'green' herbicide development, and ecophysiology. Clearly, plants compete in the natural environment, and allelochemicals help to give certain species a selective advantage over their neighbours. We have focused on the ability of cress seeds to exude substances that adversely affect the growth of neighbouring 'competitors' — in our model experiments, amaranth seedlings to facilitate comparison with much of the earlier work (Hasegawa *et al.*, 1992; Iqbal *et al.*, 2012).

It was previously established that K^+, exuded from cress seeds as a component of LCSE, acts to over-stimulate the elongation of amaranth hypocotyls and restrict their growth in girth, thus weakening them (Hasegawa *et al.*, 1992; Iqbal *et al.*, 2012). In our present work, the elongation of the amaranth hypocotyls was strongly promoted by LCSE.

Conversely, the elongation of the amaranth roots was strongly inhibited by the presence of cress seed(ling)s (Fig. 1), a highly reproducible adverse effect also initially suggested to be due to the presence of lepidimoic acid, a rhamnogalacturonan-I-derived disaccharide (Hasegawa *et al.*, 1992). However, more recent work indicated that neither lepidimoic acid nor K^+ was responsible for root growth inhibition (Fry, 2017). Indeed, later work from Hasegawa's group did not strongly support lepidimoic acid (Yamada *et al.*, 2007). The root inhibitors are organic and clearly not K^+ ions; and KCl at reasonable concentrations did not affect root growth in the present work. However, the nature of the root growth inhibitor was unknown and has been investigated here.

The present paper confirms that cell-free LCSE mimics the presence of live cress seed(ling)s and robustly inhibits amaranth root growth (Fig. 2b) and shows that the inhibitor responsible is non-volatile (not lost on drying), organic (destroyed on ashing, unlike K^+), strongly hydrophilic (thus not one of the classic phytohormones), and stable to hot acid (unlike a disaccharide). The main aim of the present paper was to characterise the root growth inhibitor(s) present in cress-seed exudate. Our working hypothesis, developed below, is that the root inhibitory principle is a mixture of small molecules.

Prior to a more detailed study of the root inhibitor(s) present in LCSE, we needed to overcome certain practical difficulties, and the findings of this part of the work will assist future studies on the active principles present in LCSE and any comparable allelochemical preparations. We tested the phytotoxicity of residues left after evaporating several solvents commonly used for chromatography and electrophoresis (Fig. S2) and found that all were acceptable except white spirit ('turpentine substitute'), which is routinely used as the coolant during paper electrophoresis in pH 2.0 buffer (Fry 2020). Even after repeated drying of white spirit, including re-drying from any of a range of other solvents, an (invisible and odourless) substance evidently remained which completely inhibited amaranth seed germination. Thus, the active principle(s) of LCSE could not be studied by electrophoresis at pH 2.0. Nevertheless, the pH 2.0 solvent itself, as well as the coolant (toluene) and buffers for electrophoresis at pH 6.5, and the paper chromatography solvent BAW, were satisfactory.

Concerning another potential artefact, we also found that K^+, the major cation present in LCSE — although not itself inhibiting root growth when added as KCl, KH_2PO_4, or K_2SO_4 — was capable of trapping otherwise volatile acids (acetic and formic) that would often be added during chromatography or electrophoresis (Fig. 3). To overcome this

problem, we found that it was possible to exchange the acetate or formate for chloride by adding a small excess of HCl and then drying it off (Fig. 3d): the great majority of the acetate and formate would then be released as volatile acetic and formic acids, the excess HCl would also be volatilised, and the non-volatile K^+ would be left as innocuous KCl.

Taking precautions to overcome the above potential problems, we were able to gain further insight into the nature of the root inhibitor(s) present in LCSE. They remained in the aqueous phase (at all pH values tested) when partitioned against butan-1-ol or toluene, and were thus highly hydrophilic. Most naturally occurring carboxylic acids lose their negative charge at pH 2, which would enhance their ability to partition into butanol. Likewise, most naturally occurring amino compounds lose their positive charge at high pH, favouring their partitioning into butanol [as, for example, sphingosine]. The inability of the root inhibitors to partition into organic solvents from water at any pH suggests that they were highly hydrophilic (e.g. sugars), and not hydrophobic weak acids or bases.

To investigate the root inhibitors' ionisation, we performed high-voltage electrophoresis of LCSE and bioassayed the fractions. Under these conditions, the hypocotyl promoter gave a very prominent peak of bioactivity co-migrating with K^+ (Fig. 4a), as expected. However, the root inhibitor — initially highly bioactive (Fig. 2b), gave only a weak zone of bioactivity on the electrophoretogram (Fig. 4b). The minority of the root-inhibitory principle that did remain statistically detectable was in the neutral region (zone 6), co-migrating with glucose and fructose (Fig. 4c). The loss of some of the activity, despite the fact that the root inhibitor did not partition into toluene (used as coolant for electrophoresis at pH 6.5), suggests that the root inhibitor comprised multiple compounds, which would synergise but were partially resolved by electrophoresis. Indeed, there was a hint of root-inhibitory activity in zones 3 and 8, albeit not strongly different ($p > 0.01$) from the mean of the four zones that permitted maximum root elongation (Fig. 4c). The idea of multiple components was supported by the total loss of LCSE's detectable root inhibitory activity upon paper chromatography (Fig. 5d), which is considered to have resolved multiple different solutes such that they were unable to act synergistically.

On gel-permeation (size-exclusion) chromatography on Bio-Gel P-2, the root inhibitor co-eluted with neutral hexoses (M_r 180), indicating that the majority of the root-inhibiting constituents of LCSE were small molecules.

In summary, although the specific components of cress-seed exudate that interfere with the root growth of neighbouring competitor seedlings remain to be identified, they are concluded to be a mixture of small organic molecules — some of them neutral (migrating in zone 6 of the electrophoretogram) and others charged (thus distributed over other zone(s) of the electrophoretogram and therefore unable to contribute synergistically to an allelopathic effect; Fig. 4) — which would all require to be present in order to exert the full biological impact on competitor seedlings. Such a mixture of small molecules might *a priori* be suggested to have its effect on root growth by building up a sufficiently high osmotic pressure (which translates into a negative water potential in the surrounding medium) to suppress water uptake and thus root cell expansion. However, this hypothesis was rejected because (a) the measured osmolality of LCSE was only 11 mOsmol kg^{-1} (equivalent to 11 mM of total sugars), and (b) the total concentration of solutes in LCSE was only ~1.6 mg/ml, which, if due to hexose monosaccharides, would equate to ~9 mM, a concentration at which glucose had no discernible effect on root growth (Table S1).

The allelochemicals found here to be exuded by cress seeds clearly inhibit root growth in neighbouring amaranth plants. Our work focused on an arbitrarily selected model system: cress *versus* amaranth. We do not expect this particular pair of plant species to frequently occur in close proximity; however, the cress-seed allelochemicals documented here may well inhibit root growth of many other species that do tend to compete for establishment in the same soil, either horticulturally or in the wild. In addition, the seeds of numerous other plant species probably also exude comparable allelochemicals. There remain many exciting opportunities to detect and characterise new allelochemicals by experiments similar to those used here.

Conclusions

Cress seeds exude a range of substances that together act as allelochemicals, interfering with the growth of neighbouring (potentially competing) seedlings — amaranth, in our model experimental system. These low-M_r substances include K^+ ions, which specifically over-stimulate hypocotyl elongation, plus a cocktail of small, hydrophilic, heat-stable, organic compounds (including, as identified by chromatography and electrophoresis, glucose, fructose, sucrose and galacturonic acid) that together inhibit root growth. These major sugars of cress-seed exudate, however, are present at too low a total concentration (even if augmented by K^+), to inhibit root growth by creating a high osmotic pressure.

Thus, the root-inhibitory allelopathic principle is not a single specific allelochemical, but rather a mixture of small organic molecules which, however, remain to be identified.

SUPPLEMENTARY DATA

Supplementary data are available online at ... and consist of the following.

Figure S1: Failure of LCSE active principles to partition from water into immiscible organic solvents.

Figure S2: Effect of residues from commonly used solvents on amaranth seedling growth.

Figure S3: Paper chromatography of low-M_r cress-seed exudate (LCSE) and some relevant markers

Figure S4: Two replicate studies of the behaviour of the active principles of LCSE on gel-permeation chromatography.

Table S1: Effects of osmotica on amaranth seedling growth

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598

599 **LEGENDS TO FIGURES**

600

601 **Fig. 1. Effect of cress seed(ling)s on germination and seedling growth of**
602 **amaranth.**

603 Amaranth seeds (10 per dish) were incubated in the dark on damp filter paper in 5-cm
604 Petri dishes in the presence of 0–12 cress seeds. After 4 days, the amaranth seedlings
605 were measured (a, hypocotyls; b, roots). The number of amaranth seeds that germinated
606 is also recorded (c). N = 61–67 dishes for each number of cress seeds tested; asterisks
607 indicate significant effect of cress seeds, ** $p < 0.002$.

608

609 **Fig. 2. Low-molecular-weight cress-seed exudate (LCSE) promotes amaranth**
610 **hypocotyl growth and inhibits amaranth root growth through two different**
611 **agents.**

612 Amaranth seeds were incubated on damp filter-paper for 4 days in the presence of LCSE
613 (total solute concentration ~1.6 mg/ml) that had been stored frozen, compared with
614 LCSE treated by: freeze-drying; ashing in a Bunsen flame; incubation with 0.25 M
615 formic acid for 1 h at 20°C or 120°C; and partitioning against ethyl acetate (acidified to
616 pH 2.7 with 20 mM formic acid; the upper hydrophobic organic phase and lower
617 hydrophilic aqueous phase were separately bioassayed). Any formic acid or ethyl acetate
618 were dried off prior to the bioassays, and all LCSE specimens were reconstituted in
619 deionised water to the original volume. The right-hand three bars represent controls
620 with no LCSE present: 'acid-only hydrophobic' and 'acid-only hydrophilic' were the
621 organic and aqueous phase, respectively, after 20 mM formic acid was shaken with ethyl
622 acetate. N = 15 Petri dishes for each treatment; ** $p < 0.002$ compared with
623 frozen/thawed LCSE. Dashed line, frozen/thawed LCSE; solid line, water-only control.

624

625 **Fig. 3. Effect of salts on amaranth seedling growth.**

626 Ten amaranth seeds per dish were incubated for 4 d in the dark in the presence of
627 various salts at (a) 5 mM or (b–d) 10 mM, then the hypocotyl and root lengths were
628 measured. Each treatment was applied in 3 Petri dishes; the histograms show the mean
629 organ lengths ± SE. In each case the germination was 70–80%. In (c), the salts were
630 prepared in-house by adjustment of 10 mM KOH to pH 6.0 with the appropriate acid. In

(d), the 10 mM KOAc was adjusted to pH 3.0 with a small excess of acetic acid, mimicking a potential 'K^+-trapped' anion present in LCSE, then dried from a 100 mM solution of the acid (or water) named on the x-axis.

The dashed line indicates the water-only control. Asterisks: * $p < 0.01$, ** $p < 0.001$ (in each histogram compared with the water-only control).

Fig. 4. Behaviour of the active principles of LCSE on electrophoresis.

Replicate samples (N = 48) of LCSE were fractionated by paper electrophoresis at pH 6.5 for 13 min at 2.5 kV. Each of the 48 electrophoretograms carried a marker mixture containing K^+, Na^+, Mg^{2+}, Cu^{2+}, XXXGol-sulphorhodamine (neutral marker, fluorescent), lepidimoic acid (Lep), galacturonic acid (GalA), Orange G, phosphate (P_i) and sulphate (SO_4^{2-}). (a) The marker mixture was cut off each of the 48 electrophoretograms together with a fringe of the neighbouring LCSE loading, and stained with bromophenol blue; 16 representative runs are illustrated. The unstained majority of each electrophoretogram was then cut into ten zones, each of which was eluted with water and the eluate bioassayed on hypocotyl growth (b) and root growth (c) of amaranth seedlings. The histograms show the mean seedling lengths (± SE; N ≈ 48). In (b), the dashed line indicates the mean of the shortest four; in (c) it is the mean of the longest four. Asterisks indicate that the specific zone significantly differed from the mean (N ≈ 192) of the relevant four zones : * $p < 0.01$, ** $p < 0.001$.

Fig. 5. Paper chromatography of active principle(s) present in low-M_r cress-seed exudate.

a,b: Paper chromatography (in BAW) of five examples out of 15 independent LCSE samples. (a) Stained with bromophenol blue, revealing ionic constituents; (b) same chromatograms stained with silver nitrate, revealing sugars. MM, Marker mixture [10 µl containing 30 mM of each of lepidimoic acid, galacturonic acid, potassium sulphate and a trace of Orange G]. 'LCSE': a 100-µl streak-loading (~4 × 1 cm) of 20-fold concentrated LCSE. Spots labelled in yellow: components of marker mixture. Spots labelled in white: components of LCSE (Fru, fructose; Glc, glucose; GalA, galacturonic acid; Sucr, sucrose; Unk = unidentified anion). [Spots are labelled only on the 4th chromatogram.] Chromatography was on acid-washed Whatman No. 3 paper, developed in butan-1-ol/acetic acid/water (12:3:5) for 20 h. After thorough drying, the paper was

rapidly dipped through methanol/acetone (1:2) and re-dried, repeated several times, and finally re-dried in a draught of air overnight, helping to remove the last traces of acetic acid.

c,d: Strips corresponding to zones 1–10 were excised from replicate 100-µl streak-loaded chromatograms (identical to a and b but not stained; not shown) of the fifteen concentrated LCSE samples; each strip was eluted into 1 ml water, and the eluates were bioassayed for effects on the growth of amaranth hypocotyls (c) and roots (d). The approximate migration positions of various markers (with some variation between the 15 chromatograms) are indicated above histogram (c).

Fig. 6. Behaviour of the active principles of LCSE on gel-permeation chromatography.

LCSE was run through a Bio-Gel P-2 column and selected even-numbered fractions were tested for (a) thymol-reactive sugars and for the ability to (b) promote amaranth hypocotyl growth and (c) inhibit root growth. Abbreviations in (a) are: B1, unidentified sugar as named by Iqbal *et al.* (2016); Fru, fructose; Glc, glucose; Sucr, sucrose; DP3, probable neutral trisaccharide; PS, polysaccharides; Mlt2, maltose; Mlt3, maltotriose. Asterisks indicate a significant effect of the fraction (mean ± SE; N ≈ 20) compared with the mean of all fractions; * $p < 0.01$, ** $p < 0.001$. The most effective fraction for root inhibition is marked with a vertical arrow.