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Detection and identification of karrikinolide in planta

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Abstract

Two decades ago, karrikins (KARs) were identified as the active constituents of smoke water, responsible for stimulating seed germination in many fire-following plant species. The most potent member KAR1 was structurally characterised 1 and influences a wide range of plant developmental processes 2. KARs are assumed exogenous molecules generated during biomass combustion and are presumed not naturally present within plant tissues. We describe the detection, identification, and purification of KAR1 from the roots of multiple plant species and show biological activity using a lettuce (Lactuca sativa) germination bioassay 3, demonstrating its capacity to promote seed germination in a comparable manner to the authentic KAR1 standard. These data challenge the long-held assumption that KARs are not present in planta. The identification of endogenously produced KAR1 suggests that KARs, or derivatives thereof, constitute a previously unrecognised endogenous signalling molecule in plants, most likely mediated by the KARRIKIN INSENSITIVE 2 (KAI2) receptor.

Main text

Wildfires and the burning of crop residues have been a long-established and important aspect of agriculture, promoting vegetation renewal, nutrient cycling and landscape regeneration [4]. During the pyrolysis of plant biomass, smoke and char are generated and comprise a complex mixture of bioactive compounds capable of stimulating seed germination. Since the early 20th century, numerous mono- and dicotyledonous species have been shown to respond positively to smoke or smoke-water treatments, including many species that do not exhibit classical fire-responsive germination strategies [5,6]. In 2004, biochemical fractionation with concurrent bioassays for seed germination identified the principal germination-promoting compound in smoke from burnt plant material as 3-methyl-*2H*-furo[2,3-*c*]pyran-2-one, commonly known as KAR_1 [1]. Subsequent studies led to the identification of five additional naturally occurring KAR analogues. KARs share a common bicyclic butenolide structure consisting of a six-membered pyran ring fused to a five-membered butenolide ring, with individual analogues differing in the number and position of methyl substituents [6].

Current evidence indicates that the formation of KARs requires both oxygen and carbohydrate-rich substrates (e.g. plant biomass or cellulose) during combustion [5,6]. However, the precise chemical mechanisms responsible for their generation remain unresolved. Furthermore, KARs have thus far been detected solely in smoke and soils shortly after fire events, supporting the longstanding view that KAR molecules arise exclusively through pyrolytic processes rather than through endogenous plant biosynthesis [7]. Consequently, despite the discovery of the KAR signalling pathway and its receptor KARRIKIN INSENSITIVE 2 (KAI2), it has been assumed that no endogenous biosynthetic pathway for KAR production is present *in planta* [8].

Genetic and biochemical studies have revealed that KAI2 is widely conserved throughout the plant kingdom, highlighting the evolutionary importance of this signalling pathway [9]. Several studies have

demonstrated that KAR_1 can interact with KAI2 [8,9]. However, structural analyses in KAI2 indicate, that KAR_1 occupies a position near the entrance of the ligand-binding pocket, limiting access to the catalytic triad and thereby restricting hydrolytic activity. This observation is particularly significant because both *in vitro* and *in vivo* studies suggest that hydrolysis plays an important role in receptor activation and signal transduction [8,10]. This finding has led to the postulation that KAR_1 itself may not represent the biologically active ligand perceived by KAI2, but rather requires metabolic conversion within the plant to generate a signalling-competent molecule [11].

An alternative, and increasingly influential, hypothesis is that plants synthesise an endogenous KAI2-ligand (KL), that shares structural and functional similarities with KARs [12]. This is supported by the characteristic developmental phenotypes of *kai2* and *max2* mutants, which exhibit signalling defects even in the absence of fire-derived compounds. Despite extensive investigation, the identity and biosynthetic origin of this putative endogenous ligand remain unknown [12].

With the aim of identifying KL molecules, metabolomic analysis was performed on root samples from barley and rice lines using LC-QTof platforms [13,14]. A molecular feature putatively identified as the KAR_1 molecule was consistently detected in barley roots and sporadically in rice roots. However, no corresponding compound was detected in shoot tissue. Furthermore, roots from several other species were assessed for the presence of KARs (Supplementary Fig. 1). The putative KAR_1 was observed in roots of five diverse barley varieties as well as wheat, maize, pea and red clover at similar quantities (~1-2 ng/g dry weight). In rice, sporadic detection of a putative KAR was evident, while in *Arabidopsis*, tobacco, pearl millet, and oat no KAR could be detected. However, instrumental sensitivity and trace endogenous levels could be a limiting factor in these species.

Deploying the Metabolomics Standards Initiative (MSI) guidelines [15], further steps were implemented to confirm the identity of the putatively identified KAR_1 with multiple analytical platforms and its biological activity. Firstly, an authentic KAR_1 standard was obtained and its chemical structure confirmed. The authentic KAR_1 was analysed under identical chromatographic conditions to those used for root extracts by LC-QTof, both independently and spiked into a barley root sample (Fig. 1). Barley and rice test material was generated independently at two environments, including production under sterile hydroponic conditions to exclude any contamination from sand/soil material. Secondly, the KAR_1 standard as well as roots from barley grown under different nutrient and arbuscular mycorrhizal fungi (AMF) conditions, which should influence KAI2 signalling, were analysed by GC-MSD as an alternative analytical platform (Fig. 2).

Following MSI guidelines for a positive identification of KAR_1, multiple characteristics such as retention time, accurate mass and mass spectra were taken into consideration and compared to the authentic standard [16]. Identical retention times for the compound present in the sample and the authentic standard were observed on both analytical platforms. The monoisotopic masses $[M+H]^+$ determined by LC-QTof differed by 80-105ppm between the sample (151.0242) and the authentic standard (151.0401)

(Fig. 1). The same accurate mass difference was observed for the spiked sample (151.0246), with an over two-fold increase in peak area compared to the unspiked barley root samples. These data represent co-chromatography and co-spectral properties between the authentic standard and the experimental samples (Fig. 1). Thus, providing positive identification of KAR present *in planta*. Small mass offsets can be explained by automated mass correction, which can be affected by an inconsistent spray of the lock mass throughout the analytical run, or the sample matrix affecting the mass centroid determination. In fact, if the compound in question had a different mass compared to the standard, then both masses should have been resolved by the resolving power (>25,000) of the LC-QTof used with an employed acquisition rate of 0.9 spectra/s [17]. Additionally, the same mass difference could be observed for the isotopic peak in the standard (152.0425) as well as the independent and spiked samples (152.0295 and 152.0284), respectively. MS/MS fragmentation of the putative compound was not possible, due to the low quantity (~15 pg/5 µL injection volume, ~3 nM) and co-elution with ~30 compounds <300 Da and ~46 compounds >300 Da. The coelution of the endogenous KAR_1 with more abundant metabolite signals as well as the concentration below µM impede the identification by NMR; and highlights the importance to explore alternative approaches [18,19].

The second analytical platform used for identification was GC coupled to a mass detector with electron impact ionisation. The EI-MS fragmentation pattern of the KAR_1 standard showed the molecular mass 150.0 together with four prominent fragment ions at m/z 121.0, 105.0, 94.0, and 65.0. This pattern was identical between the putative compound and authentic standard (Fig. 2). The mass spectrum has characteristic losses of small neutral species such as CO, CHO and ketene-like fragments, previously reported with similar fused furo[2,3-c]pyran-2-one ring system [20,21]. The fused furan and pyranone rings provide extensive conjugation, conferring relative stability to the molecular ion formed following electron impact ionisation (m/z 150). The presence of the m/z 121 fragment suggests that the initial major fragmentation event involves cleavage adjacent to the lactone carbonyl group. The neutral loss of 29 Da can be attributed to loss of a CHO moiety following hydrogen transfer and ring opening, generating a resonance-stabilised oxonium/furanyl cation. The ion at m/z 105 is consistent with subsequent loss of oxygen from the oxygen-rich heterocyclic framework after ring opening. Formation of the m/z 94 fragment may result from further ring contraction or ring opening, accompanied by hydrogen migration and loss of a carbonyl-containing fragment. The final diagnostic ion at m/z 65 could arise through loss of a second CHO group, producing a highly stabilised cyclopentadienyl- or furanyl-type cation ($C_5H_5^+$). Fragments in this mass range are common terminal products of the fragmentation of oxygen-containing five-membered heterocycles because they retain aromatic stabilisation [20].

As an alternative approach for identification, the biological activity of the endogenous KAR_1 was assessed with an established germination assay using lettuce [22]. To generate sufficient quantity of KAR_1, root material from two hundred barley plants was pooled for solvent-based extraction, enrichment by SPE and purification through multiple HPLC runs. To exclude any biological effects of the chromatographic solvents, eluent of solvent injections was collected over the same period as the endogenous KAR_1 and used as a comparator. The final concentration of the purified endogenous KAR_1

(0.7 nM) was defined by the quantity extracted from the root material and volume needed for enough replicates to ensure sufficient statistical power. For the bioassay, a germination rate for different concentrations of the authentic KAR_1 standard was established (Fig. 3). Treatment with 1 nM KAR_1 resulted in a germination rate of 50.8%, which was a significant ~30% increase compared to the untreated control with 39.1% germination (Tukey HSD, *P*adj = 0.0103). The purified endogenous KAR_1 (0.7 nM) achieved a germination of 42.4%. This result was lower than expected from the germination rate of the standard, but significantly higher than the control fraction with a germination of 31.4% (Tukey HSD, *P*adj = 0.0046) (Fig. 3). The lower germination rate of the control fraction compared to 0 nM KAR_1 suggested the presence of germination inhibitors, probably related to solvent residue. Regardless of these suppressing compounds, the purified endogenous KAR_1 displayed stimulation of germination similar to the authentic standard.

The structural similarities between KARs and strigolactones have prompted speculation regarding possible biosynthetic relationships. A notable difference between the species containing the putative compound and the other species analysed was their preference for the biosynthesis of non-canonical strigolactones (SL), which along with canonical SL are the ligands of D14. Thus, one hypothetical pathway for the formation of KAR could involve oxidation of a strigolactone precursor followed by ring contraction to generate a KL scaffold. However, this possibility is not strongly supported by experimental evidence as correlation analysis was performed for strigolactones and KAR_1 detected in three different barley panels containing mutants impaired in the transcriptional regulation of *Nodulation Signalling Pathway* (*NSP*) for the regulation of AMF symbiosis [13] grown under different phosphate conditions. The data indicated no correlation (R^2<0.05 and *P*>0.05, Supplementary Fig. 2) between KAR_1 and the strigolactone heliolactone, indicating different metabolic pathways for the biosynthesis of these two compound classes [2,23,24]. Furthermore, barley roots were harvested at four, five, six and seven weeks after potting and contained fluctuating levels of KAR_1 (respectively 2.6, 3.3, 1.9 and 2.9 ng/g DW). These levels are contrary to expected levels of strigolactones [25] and support the hypothesis of two biosynthetic pathways.

Even though some structural similarities can be drawn with sesquiterpenes derived from the same precursors as strigolactones, KAR is a comparatively small fused-ring molecule. It therefore seems unlikely that a biological system would first construct a C_{15} sesquiterpene scaffold only to extensively degrade it to produce a C_8 butenolide. Furthermore, KARs lack the characteristic methyl branching patterns typical of isoprenoid-derived metabolites and their high oxygen-to-carbon ratio is mostly observed for highly modified terpene products, e.g., loliolide [26,27]. An alternative and chemically more plausible route for KAR biosynthesis could involve a polyketide/α-pyrone precursor. Type III polyketide synthase (PKS) or 2-pyrone synthase-like enzymes utilise acetyl-CoA and malonyl-CoA as precursor molecules to generate an α-pyrone intermediate [28,29]. Subsequent steps would include fusion of the ring system through oxidative cyclisation catalysed by a P450 enzyme, which have been previously reported in natural product biosynthesis [30]. Future isotope-labelling studies could help resolve the biosynthetic

origin of KAR. Incorporation of ^{13}C-acetate into KAR would support a polyketide-derived pathway, whereas incorporation of ^{13}C-glucose with retention of an intact carbon skeleton would support a carbohydrate-derived route. Overall, a PKS-based biosynthetic pathway provides the most parsimonious explanation, should KAR prove to be products of enzymatic biosynthesis.

In summary, KAR_1 was detected only in roots of numerous mono- and dicot plant species, contrary to the long-held assumption that KARs are not formed by plants. This result could be a combination of instrument sensitivity at the time as well as the plant species/tissue tested. Previous studies demonstrated the diverse roles of KAI2 and KARs signalling in plant biology [5,6]. Endogenous biosynthesis of KAR-like molecules could lead to the discovery of more effective plant growth-promotors facilitating seed priming, stress resilience, and biomass enhancement. Unravelling their biosynthetic pathways presents a transformative opportunity to advance both fundamental plant science and agricultural innovation, contributing to more productive, resilient, and sustainable food systems worldwide.

Methods

Plant materials and growth conditions

Plants cultivated with sterilised quartz sand (grade 14/25, Aggregate Industries, UK) included *Arabidopsis thaliana,* barley (*Hordeum vulgare* L. cv. Golden Promise), maize (*Zea mays* L.), oat (*Avena sativa*), pearl millet (*Pennisetum glaucum* L. R.Br.), rice (*Oryza sativa* cv. Nipponbare and Shiokari), tobacco (*Nicotiana tabacum* L. K326) and wheat (*Triticum aestivum* L. cv Fielder).

Hydroponically grown plants included barley, rice cv. Nipponbare and Shiokari, pea shoots (*Pisum sativum L.) and* red clover (*Trifolium pratense* L.).

Seeds for both sand and hydroponic growth conditions were sterilised and propagated as previously described [13]. Seedlings were transferred to a cone system (CONE-TAINERS™, Stuewe & Sons) containing sterilised quartz sand (~70 g) and watered with reverse osmosis (RO) water three times a week, for the first two weeks. Subsequently, plants were fertilised with half strength Hoagland solution under low phosphate conditions (10 µM Pi) [31]. Roots and shoots were harvested six weeks after transplantation and flash frozen in liquid nitrogen before lyophilisation. For the time course, barley roots were collected after four, five, six and seven weeks.

Hydroponic conditions were performed as previously described for rice in 50 ml falcon tubes [31]. Half strength Hoagland solution (400nM Pi) was refreshed three times a week for the first two weeks. Subsequent feeding was performed with reduced phosphate (4nM Pi) for one week. Roots were dried with paper towels, cut at the edge of the tubing and lyophilised.

Metabolite extraction and analysis

All samples were lyophilised and ground to a fine powder before further processing. The extraction method was adapted and optimised for low amounts (10 mg) of dried material [31,32]. The root and shoot samples were dissolved in cold 60% acetone (v/v, 0.5 mL) and mixed for 30 min at 40 rpm and 4°C in dark conditions. Samples were then centrifuged for 5 min at 20,000*g* and 4°C. The supernatant was recovered and dried under vacuum. The dried extract was re-dissolved in 10% acetonitrile (v/v, 0.5 mL) and concentrated in an SPE cartridge Oasis HLB Prime 1cc (30mg) (Waters) using a vacuum manifold apparatus (Macherey-Nagel). Compounds were eluted with acetone (0.5 mL) and dried under vacuum. Before analysis, samples were dissolved in 25% acetonitrile (v/v, 50 µL).

The KAR_1 standard was purchased from Chiralix (Symeres Netherlands B.V.) and resuspended in acetone (1 mg/mL) before serial dilution with 25% acetonitrile (v/v).

Liquid chromatography analysis was performed with an Agilent 1290 Infinity II (Agilent Technologies, Inc.) coupled to an Agilent 6560 Ion Mobility QTOF in positive ESI mode. Samples (10 µL) and the authentic standard KAR_1 were separated with an ZORBAX Eclipse Plus C18 50x2.1 mm i.d, 1.8µm (Agilent Technologies, Stockport, UK) at a flow rate of 0.3 mL/min. Mass range was 80-1000 m/z and the acquisition rate at 0.9 spectra/s and acquisition time of 1111.1 ms/spectrum was used.

Gas chromatography was performed with an Agilent GC 8860 coupled to a 5977C mass selective detector (MSD) based on a previous method [33]. The heat gradient was changed to 8°C/min. Samples and authentic standard were injected (1 µL) without derivatisation and the same dilution as used for LC-QTof analysis.

Data extraction and visualisation of chromatograms and mass spectra were performed with Agilent MassHunter Qualitative (V10.0). Molecular structures were created with ChemSketch (2024.2.3, ACD/LABS).

KAR purification from barley roots

Barley roots were lyophilised and ground into fine powder with TissueLyser II (Qiagen). Aliquots (10 g) were extracted with 60% (v/v) acetone (500 mL) for 2 hrs at 4°C, covered in aluminium foil and agitated with a magnetic stirrer. Extracts were filtered under vacuum with a Buchner funnel and Whatman 1 filter papers (Cytiva, 1001-125) and partitioned with ethyl acetate at a ratio 2:1. The top partition was concentrated with Hei-VAP Ultimate laboratory rotary evaporators (Heidolph Instruments), resuspended in 25% (v/v) acetonitrile: water (OPTIMA® LC-MS grade, Fisher Scientific) and separated by LC-QTof as described above. The KAR fractions were pooled and dried down under nitrogen gas.

Lettuce seed germination assay

Lettuce germination assays were conducted as previously described with minor modifications [3]. Before use, seeds of lettuce cv. *Grand rapids* were incubated in dry conditions at 28°C for at least three days in the dark. For each assay, 9 cm Petri dishes were lined with two layers of 9 cm Whatman paper and

moistened with 2.5 mL sterile ultrapure water containing either a pure KAR_1 standard, enriched KAR_1 fractions from barley roots, the comparator, or a pure acetone control. This resulted in a final concentration of 0.065% acetone (v/v). All work with lettuce seed was conducted in the dark, illuminated only by a 730 nm LED light source (KingBrite, QB11 XP-E2). Approximately 50 achenes were added to each plate. Plates were sealed with parafilm and incubated at 28°C in darkness for one hour, then exposed to the 730 nm LED light source for 30 min. Germination was assessed after a further 24 hrs incubation at 28°C, where germination was assessed by the appearance of the radicle.

Declarations

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Author contributions

M.D., T.I. and E.J.B. cultivated plant material.

M.D. and E.J.B. conducted the experiments and analysed the data.

M.D., L.P.F., E.J.B., U.P, G.E.D.O and P.D.F. designed the experiments.

U.P., G.E.D.O. and P.D.F. secured the funding.

M.D., E.J.B. and P.D.F. wrote the paper, with input from all the authors.

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Figures

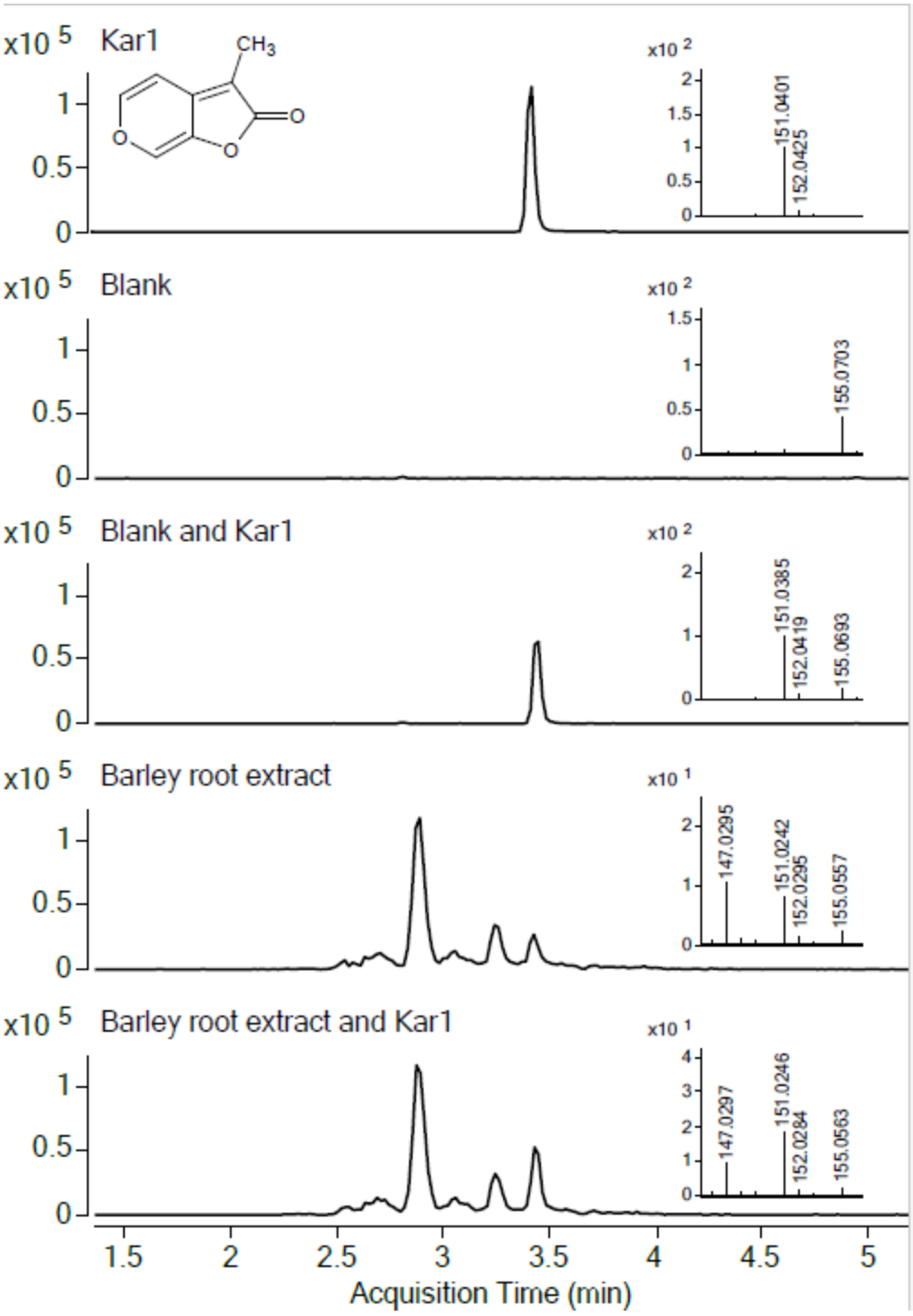


Figure 1

Chromatography of KAR_1 standard and endogenous KAR_1 in barley root samples. Extracted ion chromatograms (m/z 151.0400) were detected by LC-QTof for KAR_1, a blank solvent run and barley root extract sample pure and spiked with KAR_1 standard. Sample description is in the top left corner of each chromatographic trace and the m/z values of the peak at 3.45 min are displayed on the right side.

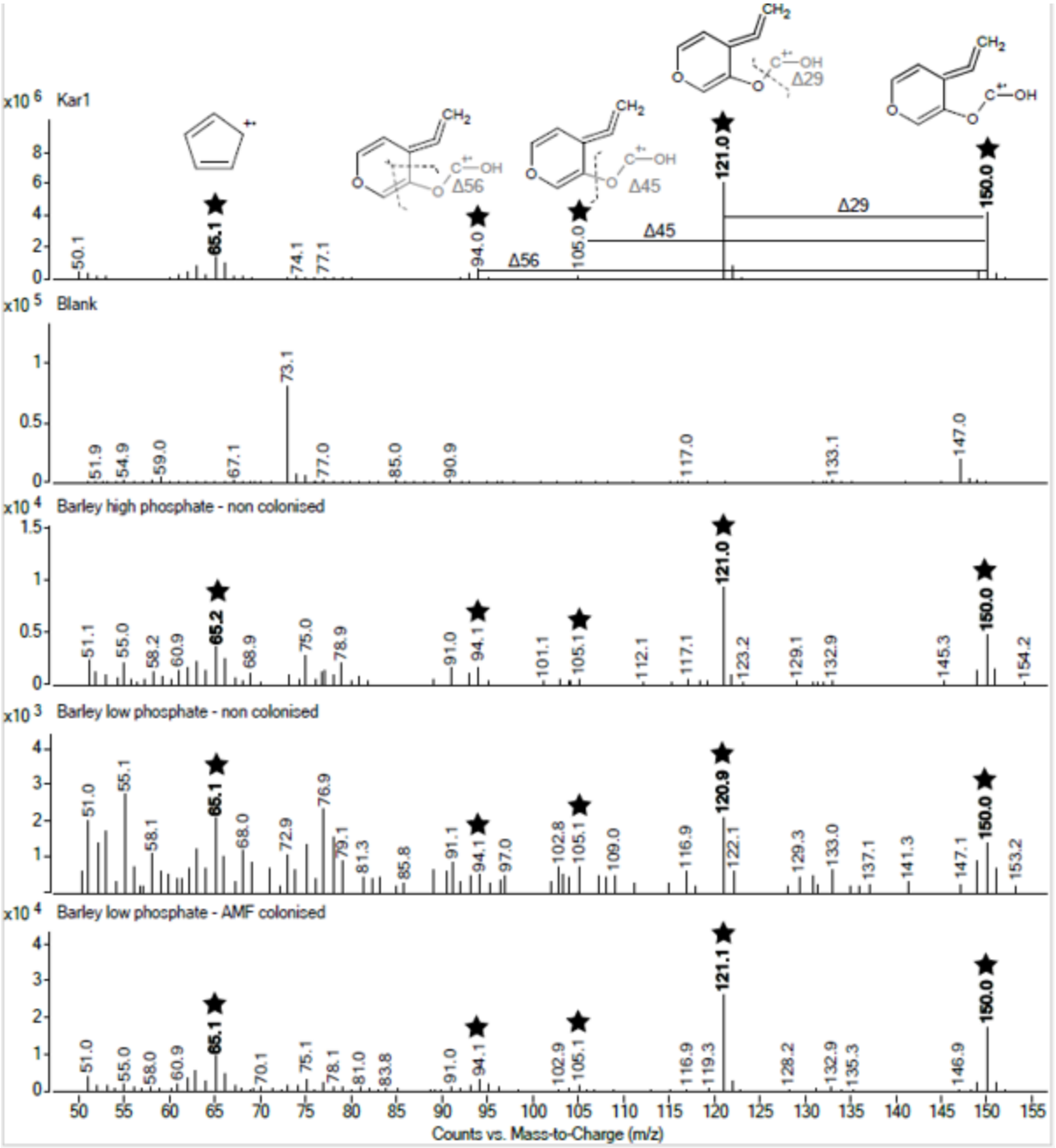


Figure 2

Mass spectra of KAR_1 standard and KAR_1 in barley root samples analysed by GC-MSD. Sample description is in the top left corner of each mass spectrum. Characteristic fragments and the molecular ion are indicated with black stars. Putative structure of the molecular ion (150.0) and predicted fragmentation based on the mass difference (Δxx) are displayed at the top. Dashed lines indicate the predicted fragmentation site in the parent ion.

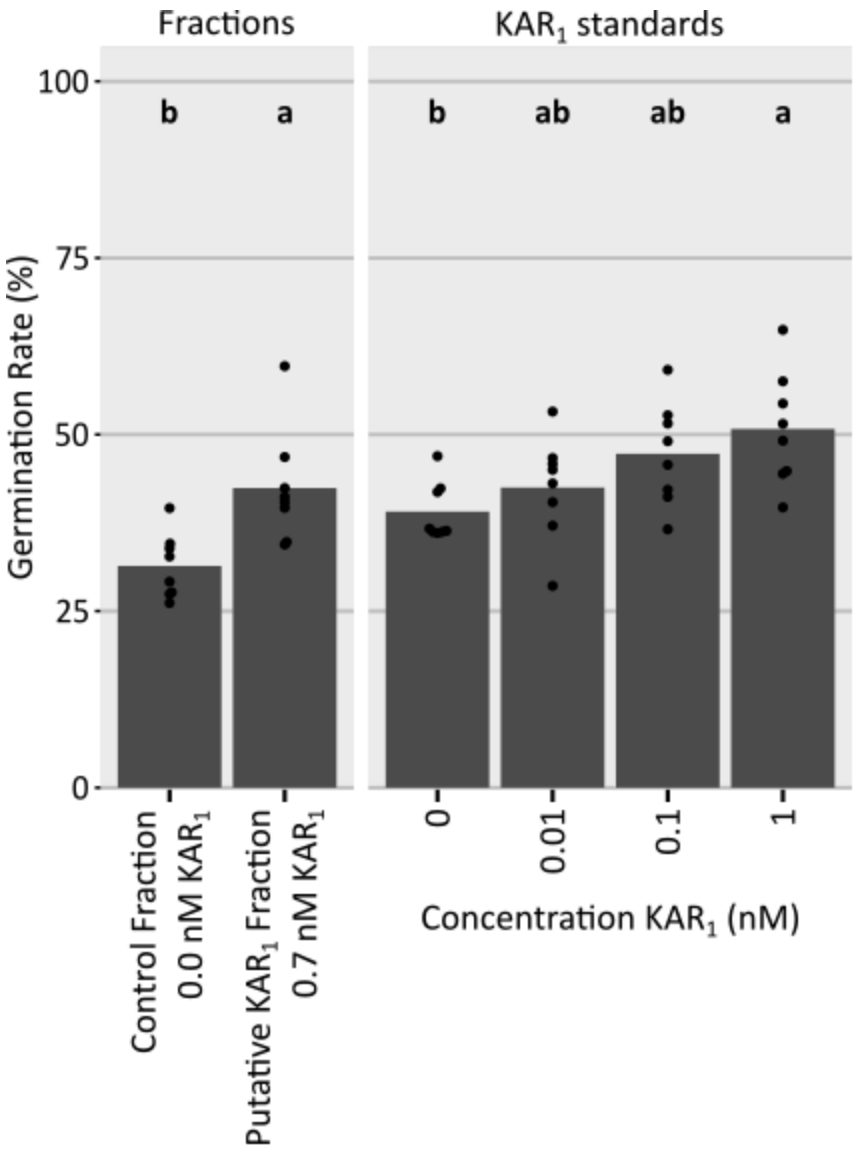


Figure 3

Germination assay of lettuce seeds. Germination rate of lettuce seed treated with pure KAR_1 standards and enriched fractions derived from plant root extracts. Statistics were calculated independently for each panel (Tukey HSD, $P \leq 0.05$). Eight plates of c. 50 lettuce achenes were assessed per condition, with each plate considered as a single replicate.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SuppFig2Correlationdata.pdf
- SuppFig1Allspecieschromatograms.pdf