Fulvic acid increases forage legume growth inducing preferential upregulation of nodulation and signalling-related genes

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Abstract

The use of potential biostimulants is of broad interest in plant science for improving yields. The application of a humic derivative called fulvic acid (FA) may improve forage crop production. FA is an uncharacterised mixture of chemicals, and although it has been reported to increase growth parameters in many species including legumes, its mode of action remains unclear. Previous studies of the action of FA have lacked appropriate controls, and few have included field trials. Here we report yield increases due to FA application in three European *Medicago sativa* cultivars, in studies which include the appropriate nutritional controls and hitherto unused. No significant growth stimulation was seen after FA treatment in grass species in this study at the treatment rate tested. Direct application to bacteria increased *Rhizobium* growth and in *Medicago sativa* trials root nodulation was stimulated. RNA transcriptional analysis of FA-treated plants revealed upregulation of many important early nodulation-signalling genes after only three days. Experiments in plate, glasshouse, and field environments showed yield increases, providing substantial evidence for the use of FA to benefit *Medicago sativa* forage production.

Highlight – Fulvic acid treatment increases yield and nodulation in *Medicago sativa*, in glasshouse and field experiments. *De novo* transcriptome analysis shows the upregulation of early nodulation genes in response to fulvic acid.

Introduction

Forage grasslands represent 26 % of global land area, and 70 % of agricultural land (FAO and IFIF, 2010). In temperate climates forage crops are cultivated and these are usually grasses (*Poaceae*) or herbaceous legumes (*Fabaceae*). The globally important legume lucerne or alfalfa (*Medicago sativa*) is of prominence in temperate forage production. For forage growers, increasing the crop's yield is a primary focus and new management practices to maintain or increase growth with lower nitrogen inputs are needed. The application of a humic substance (HS) derived biostimulant called fulvic acid (FA) may improve forage crop production.

Extractable HS fractions are considered to be key soil components and their complex composition may be responsible for facilitating many complex chemical reactions in soil systems (Canellas *et al.*, 2010; Lamar *et al.*, 2013; Sutton and Sposito, 2005; Trevisan *et al.*, 2010a). Identification of the specific effects of HS requires the use of well-structured, specific methods. Research on FA is often limited by chemical characterisation and frequently uses samples which are not easily replicable, because their source is not unique (Pandeya *et al.*, 1998). This makes designing appropriate controls for experiments difficult. Many studies often rely on a 'no application' or 'water treatment' as controls to determine the potential biostimulant effect of FA on a plant (Calvo *et al.*, 2014). In the model plant *Arabidopsis thaliana* and many cereal crops, HS has been shown to have effects on plant growth including increased root growth, improved nutrient uptake and yield under stress and control conditions, and enhanced access to metals (Bezuglova *et al.*, 2017; Dobbss *et al.*, 2007; García *et al.*, 2012; Pandeya *et al.*, 1998; Pinto *et al.*, 2004; Vaccaro *et al.*, 2015; Zhang *et al.*, 2015; Zhimang *et al.*, 2001). A study of particular importance to forage crops is that of the legumes, soybean (*Glycine max*), peanut (*Arachis hypogaea*) and clover (*Trifolium vesiculosum*) (Tan and Tantiwiramanond, 1983). This study showed that a sand growth medium supplemented with FA reduced the number of nodules whilst increasing the nodule weight in a dose-dependent manner. Application of HS to *Pisum sativum* also increased root nodulation and mycorrhizal colonisation (Maji *et al.*, 2017). If such increases were able to improve N fixation in legumes, then this could increase the N storage of the vegetative tissue and perhaps the protein content. The important forage crop *Medicago sativa* has been found to increase in vegetative biomass after FA application but with variable responses (Little *et al.*, 2013; Little *et al.*, 2014). Another study linked bulk soluble HS fractions to increased biomass of *M. sativa* and moreover nodulation with stimulated *Sinorhizobium* growth, but this study did not include nutritional controls and compared HS application to no addition (Xu *et al.*, 2018). Studies using various

HS including FAs have been carried out in other important legumes and forage grasses (Aydin *et al.*, 2012; Chang *et al.*, 2016; Daneshvar Hakimi Maibodi *et al.*, 2015; Traversa *et al.*, 2014; Verlinden *et al.*, 2010); however again no nutritional controls were used. Clearly, more detailed studies are required to fully assess the effect of FA on forage crops.

In recent years, RNA-sequencing (RNA-seq) has transformed from an exclusive tool used in discrete studies (Marguerat and Bähler, 2010; Wang *et al.*, 2009) to a critical technique accessible for many projects to investigate phenotypic changes occurring in specific conditions (Costa-Silva *et al.*, 2017). Changes in transcriptional expression in plants following stimulus, stress, or treatment can reveal the downstream signalling and metabolic changes that cause a phenotype. RNA-seq is an incredibly robust and sensitive tool (Conesa *et al.*, 2016; Martin *et al.*, 2013; Mortazavi *et al.*, 2008; 't Hoen *et al.*, 2008), providing a wealth of data that can give an understanding of the underlying mechanisms underpinning a specific treatment.

Although a potential link between HS application and increased biomass and legume root nodulation has been demonstrated, the mechanism for the condition remains unknown. Previous studies have suggested wide-ranging modes of action for this biostimulant, including a hormone-like response by plants following HS addition (Canellas and Olivares, 2014; Nardi *et al.*, 1994; Russell *et al.*, 2006; Trevisan *et al.*, 2010a; Trevisan *et al.*, 2010b), but there is a lack of transcriptional evidence to support this idea. Therefore, we have investigated the transcriptional changes that may occur in plants following FA treatment in both shoot and root tissues using RNA-seq analysis.

Two commercial FA formulations were tested in a range of important temperate forage crops including legumes, with *M. sativa* cultivars showing a stimulatory response to the application. In order to include appropriate nutritional controls for FA, chemical analyses of the commercial products were carried out. Treatments were first assessed in the glasshouse and controlled environment room to establish growth effects on crops, and in *M. sativa* to establish a nodulation phenotype. Transcriptional changes were investigated for one FA treatment compared to its nutritional control, with *de novo* assembly and annotation of RNA-seq data, designed to provide evidence for the mode of action of FA linked to yield increases. Field trials were implemented at UK forage grower sites with applications and management using industry standard practices. The aim was to identify if a change in management practice including FA treatments can increase yield in forage cultivation under conventional farming methods.

Materials and methods

Chemical analysis

Two fulvic acid materials (FAs) were acquired, VitaLink Fulvic (sourced from Holland Hydroponics & Horticulture, UK (HydroGarden Wholesale Supplies Ltd., 2016)) and MPXA (F.A.R.M. Co., California, USA (F.A.R.M. Co., 2017)). These were termed VFA and MFA for subsequent work. The soluble dry weight of each FA was determined, and the elemental composition of solutions for total N, C and trace elements was measured using inductively coupled plasma-optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS), performed for VFA at Computational and Analytical Sciences, Rothamsted Research, Harpenden, UK and for MFA at Biological Services, UEA, Norwich, UK. Samples (0.01 g/mL) were analysed by Gas Chromatography Mass Spectrometry (Agilent GC-MS Single Quad Mass Spectrometer (7890/5977), Agilent technologies, California, USA) and run information was as follows: samples were derivatised with MSTFA (Sigma-Aldrich 394866) on Ultra 2 column (19091B-102; length 25 m, internal diameter 0.2 mm, film 0.33 μm; Agilent technologies); carrier gas hydrogen at constant flow of 1.2 mL/min; inlet temperature 250 °C; injection volume 1 μL; injection mode split–splitless (30:1); oven temperature initial temperature 170 °C with ramp 10 °C/min to 300 °C and hold 300 °C for 5 min, with equilibration time: 0.5 min and auxiliary temperature: 250 °C; acquisition mode: SCAN between 50-800 m/z. Data was acquired with Agilent Masshunter Qualitative Analysis (B.07.00) and peaks were identified with NIST Atomic Spectra database (v14, National Institute of Standards and Technology, Maryland, USA) (P.J. Linstrom and Mallard, 2018) (Guijas *et al.*, 2018; Smith *et al.*, 2005). Samples were run with standards to confirm contents. Data from this analysis are in Supplementary information: ICP in Supplementary Tables S1-S2; GC-MS in Supplementary Figures S1-S2. Data was used to produce elemental controls for FAs to use in plant and microbial assays, called VC for VFA and MC for MFA as listed in Supplementary Tables S3-S4.

Nuclear Magnetic Resonance (NMR) was carried out to elucidate the type and ratio of functional groups present in FAs. [1]H- decoupled [1]H- [13]C cross polarization (CP) and CP single pulse (CPSP) solid state NMR experiments were performed at 20 °C using a 7.05 T Bruker Avance III spectrometer equipped with a 4 mm triple resonance probe operating at frequencies of 300.1 MHz (^{1}H) and 75.5 MHz (^{13}C). Each sample was packed into a zirconia rotor, sealed using a kel-f drive cap, and spun at 12 kHz. A CP contact time of 1 ms and relaxation delay of 5 s were employed, with 90° pulses of 3.5 and 4.5 μs used for ^{1}H and ^{13}C, respectively. All

spectra were referenced with respect to TMS (Sigma-Aldrich, 87920). Peak areas were obtained from the CPSP experiments (i.e. containing both rigid and mobile components) using the automatic integration tool of TopSpin 3.6.1. Subsequently, they were normalised to relative areas and grouped into different functional groups according to the expected chemical shift regions for soil organic matter (Mathers *et al.*, 2007; Mathers and Xu, 2003). This is, alkyl C (0-50 ppm), methoxyl C (50-60 ppm), carbohydrate C (60-90 ppm), di-O-alkyl C (90-110 ppm), aryl C (110-142 ppm), phenolic C (142-160 ppm) and carbonyl C (160-200 ppm). Data from NMR analysis are available in Supplementary Figures S3-S4.

Plant growth conditions

Three cultivars of *M. sativa* were tested; cv. Daisy (DLF Forage Seeds, DK), cv. Luzelle (Oliver Seeds Ltd. (bred by INRA/Agri-Obtentions, FR, 1993)), and cv. Gea (DLF Forage Seeds, DK). The forage grass *Lolium perenne* cv. AberMagic (bred by IBERS - ABY-S562-2016), was also included.

$M. sativa$ seeds were scarified prior to sterilisation with concentrated H_2SO_4 followed by six washes of sterilised deionised water (dH_2O). Seeds were then sterilised with a 10% (v/v) sodium hypochlorite solution containing 0.05% (v/v) Triton X-100 (X100) followed by six dH_2O washes. The final wash included Nystatin 5 µg/mL (Sigma-Aldrich N6261), Amoxicillin 50 µg/mL (Sigma-Aldrich A8523) and was filter sterilised to reduce fungal or bacterial contamination. The seeds were imbibed in this solution at 30 ± 1 rpm for 2 h at 4 °C, and the wash replaced for a repeat imbibing period. *L. perenne* seeds were surface sterilised with 70% ethanol. All seeds were washed in dH_2O and plated on water agar (3 g agar (AGA03, Formedium Ltd. Norfolk, UK) in 200 mL dH_2O). Seeds plates were vernalised for two days at 4 - 6 °C before being transferred to a controlled environment room with temperature at 23 °C and photoperiod of 16 h light (90 µmol m^{-2} s^{-1}) / 18 h dark. Plants were germinated before transplantation to glasshouse.

Additional vegetative growth experiments (larger screen and plate environment)

The details of two additional vegetative yield experiments with FA treatment are available in Supplementary data. For all growth experiments, FA or elemental controls were applied at the FA manufacturer's recommended rate (1% in distilled water, 10 mLs applied to the pot soil surface). This dosage rate was also used in the field trials and is therefore agriculturally

relevant. Firstly, a full screen of forage crops grown in glasshouse was undertaken for VFA and MFA in comparison to dH_2O only, see Supplementary Figure S5. Secondly, a *M. sativa* cv. Daisy screen on plates (without transfer to soil), both with and without inoculation with *Sinorhizobium meliloti* was undertaken, see Supplementary Figure S6.

Sinorhizobium colony forming unit counts

Sinorhizobium meliloti 1021 kindly provided by Anne Edwards (Metabolic Biology, John Innes Centre), was preincubated in 100 mL TY media for 2 days at 28 °C shaking at 200 rpm to full cell density (OD 600 nm = ~ 2.5), and then diluted for exponentially growing cultures to inoculate flasks for OD 600 nm = 0.1. Treatment flasks of 100 mL TY were set up as follows: NA = no addition; dH_2O = 10 mL dH_2O; VFA/MFA = autoclaved 10 mL of 10 % VFA or 5 % MFA; VC/MC = 10 mL of 10 % VC or 5 % MC. Flasks were inoculated with 10 μL of strain and incubated at 28 °C. At timepoints of 0, 1, 2, 3, and 4 days dilutions from treatment flasks of 10^{-1} to 10^{-10} were taken in triplicate and 10 μL of diluted samples was spotted onto TY agar plates. Plates were incubated at 28 °C for one day until single colonies had formed in a dilution of ~ 20 – 200. Rhizobial cell density was calculated for dilution factor and total volume of culture. The whole experiment was repeated in triplicate.

Statistical analyses

Statistical analyses of measurements across triplicate experiments were calculated in Excel® 2016, with Student t-Test for one-tailed distribution with homoscedastic data ran between VFA/VC and MFA/MC; p-value denoted with * < 0.05, ** < 0.01, *** < 0.001. Significance between treatments was shown with letters using one-way ANOVA with Tukey testing in GenStat® 18th Edition (VSN International). Graphs were designed in RStudio.

RNAseq plant material

Seeds of *M. sativa* cv. Daisy were sterilised and sown in full seed trays (36 x 22 x 6 cm) containing Church farm soil at a rate of 20 kg ha^{-1}. Trays were watered every 3 - 4 days, and at day 12 were treated with autoclaved 1 % VFA or 1 % VC to the soil at the base of the plant; VFA was compared in transcriptome analysis to VC due to it large response in both greenhouse and field trials. Plants were sampled for RNA at days 12 and 15, referred to as day 0 and day

3, respectively in subsequent analysis. For each sample ten biological replicates were pooled, with shoot and root tissue separated to provide three experimental replicates from three trays. Tissue was immediately frozen in liquid N_2 and stored at -80 °C.

RNA extraction and sequencing

Total RNA was extracted using the TRI Reagent (Sigma-Aldrich, 93289) method with phase separation using 1-bromo-3-chloropropane and precipitation with with 400 µL isopropanol and 400 µL of High Salt Precipitation solution: 0.8 M sodium citrate and 1.2 M NaCl. After incubation for 5 min at ~ 23 - 26 °C, and centrifugation at 12 000 g at 4 °C for 15 min, the pellet washed with 1.5 mL EtOH 70 % (v/v). Samples were air-dried for 5 min and contaminated DNA removed using RNase-Free DNase Set (QIAGEN Ltd. 79254).

Samples were purified using the RNeasy MinElute Cleanup Kit (QIAGEN Ltd., 74204) and initial quality checked (Supplementary Table S5). Samples were diluted to 50 − 500 ng/µL.

Library construction (poly(A) mRNA) and sequencing was performed by Novogene (HK) Company Ltd. (Hong Kong) using Next® Ultra™ RNA Library Prep Kit (New England BioLabs Inc., E7530L) and sequenced on one lane of a HiSeq™ 2000 (Illumina, HWI-ST1276) in High Output mode using 150 bp paired end reads and V2 chemistry; sequencing quality check is shown in Supplementary Table S6.

Read alignment and differential expression analysis

De novo transcriptome assembly was performed with Trinity (Grabherr *et al.*, 2011), which used all samples generated. A total of 630599 transcripts were preliminary identified, including isoforms (Supplementary Table S7). BUSCO (Kriventseva *et al.*, 2015) was ran to check benchmarking of the assembly using Universal Single-Copy Orthologs. Kallisto (Bray *et al.*, 2016) was used to align the assembly which is less subjective than ballgown mapping, providing both Transcripts Per Million (TPM) and Reads Per Kilobase Million (RPKM) for subsequent analysis.

Differential gene expression was performed for shoot and root tissue independently using Degust (Powell, 2014) with all read alignments. Tissue samples were grouped into treatment and timepoint. Transcripts with both an absolute log fold change of 0.585 (1.5 x fold change) and a false-discovery rate (FDR) adjusted p-value (q-value) < 0.05 were considered as

differentially expressed (DE) (Supplementary Figure S7). For every grouping of tissue samples, all 3 experimental replicates were required to fit these criteria, thus ensuring a very high benchmark. DE was checked using voom/Limma method (Law *et al.*, 2014) for log Fold Change (logFC) between treatments (VC, VFA) at both timepoints (0, 3). Differential expression was then checked for individual treatments between timepoints. To eliminate any differences caused by random chance or plant development changes over the 3-day timescale, transcripts that were DE based on VFA treatment alone were calculated by subtracting 0VC vs 0VFA and 0VC vs 3VC from 0VFA vs 3VFA (Supplementary Table S8).

GO term identification, functional annotation and enrichment testing

Root DE transcripts were imported into the Blast2GO v1.4.4 programme pipeline (Conesa *et al.*, 2005; Gotz *et al.*, 2008) as FASTA contigs for functional annotation. DE transcripts were checked against NCBI's non-redundant NR database (Pruitt *et al.*, 2005) with a BLAST expectation value cut-off of $1.0E^{-3}$, and hits excepted for no more than 20 sequences. Mapping was run with the EMBL-EBI InterPro library (Mitchell *et al.*, 2019) using amino acid mapping (Carbon *et al.*, 2009) with all families, domains, sites, and repeats available tested. Annotation of mapped results was run using Gene Ontology Annotation Version 2019 (The Gene Ontology Consortium, 2019; The Gene Ontology Consortium *et al.*, 2000) with the strict parameters; Annotation cut off of 55; GO weight of 5 only; E-value-Hit-Factor restricted to $1.0E^{-6}$; Hit filter set to 500; Evidence Codes weighted from 0.5 to 1 depending on depth of evidence (default software parameters). The inbuilt statistical wizard in Blast2GO was used to generate distribution graphs for sequences and hit species, shown in Supplementary Figure S8. To quality check a manual BLAST algorithm was performed (Altschul *et al.*, 1990; Altschul *et al.*, 1997; Camacho *et al.*, 2009) with NCBI database (Benson *et al.*, 2013; Benson *et al.*, 2005; NCBI Resource Coordinators, 2016; Zaretskaya *et al.*, 2008) of the 20 most significantly up- or down-regulated genes. Any genes lacking a GO annotation through InterPro were checked for annotation in QuickGO (Binns *et al.*, 2009) and UniProt (The UniProt Consortium, 2018) and added to the analysis; functional annotations can be found in Supplemental Table S9, with a graph representing top 20 Biological Process GO terms shown in Supplemental Figure S9.

To test for enrichment of different categories of *de novo M. sativa* DE transcripts relative to all expressed transcripts found in *M. truncatula* (as the closest relative), the PANTHER Classification System v14.1 was used (Mi *et al.*, 2010; Thomas *et al.*, 2003). GO-Slim graphs

were generated for molecular function, biological process, and protein class, and then an Overrepresentation test was performed using the Fisher's exact test (Thomas *et al.*, 2006), see Supplemental Table S10.

Following RNA-seq root analysis, qRT-PCR was used to measure expression of a subset of DE transcripts. A subset of seven genes was chosen to confirm with qRT-PCR. Primers were designed for genes using available *M. truncatula* sequences, and primer calculated to have 90 – 115 % efficiencies were used in qRT-PCR.

Root RNA underwent cDNA synthesis using SuperScript™ II Reverse Transcriptase (Invitrogen™ 18064022, Life Technologies Ltd.) with oligo(dT) (Invitrogen™ 18418012, Life Technologies Ltd.), and qRT-PCR was performed with SYBR® Green JumpStart Taq ReadyMix (Sigma-Aldrich); details can be found in Supplementary Table S11. Expression of the genes of interest were calculated using the arithmetic mean Ct according to analysis for $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) using the reference gene *ACTIN2,* as expression variance was comparable across all samples. Mean relative expression was calculated for experimental replicates and compared to RPKM logFC of DE transcripts.

Field trials

To assess if yield increases in *M. sativa* from VFA found in both plate and glasshouse experiments was applicable to growers, field trials were carried out over 2017 and 2018 growing seasons. In 2017, trials were performed at Dengie Crops Ltd. (Southminster, Essex) with cv. Daisy and Fado. In 2018, the trials were at both Blankney Estates Ltd. (Blankney, Lincolnshire) and A Poucher and Sons (Bardney Dairies) Ltd. (Market Rasan, Lincolnshire) with the cv. Daisy and Gea respectively.

Treatments to be tested were NA, dH$_2$O, 1 % VFA and 1 % VC. Individual experimental design of each plot is shown in Supplementary Figure S10. Each trial contained 4 - 6 plots per treatment of areas 4 - 10 m^2 with buffer zones between plots. As in the glasshouse trials treatments were applied and at 21 days post treatment samples were taken for vegetative biomass measurements using a randomised 625 cm^2 area. Samples were also taken for protein and chlorophyll for 2018 trial plots and analysis carried out at British Chlorophyll Company Limited (Blankney Estates, Navenby); protein was detected using the Kjeldahl method, and chlorophyll using a Soxhlet extraction (Supplemental Figure S11).

Results and discussion

Analysis of FA found varying chemical composition

FAs were analysed for their elemental content using a range of techniques, with results shown in Supplemental Tables S1-S2 and Figures S1-S4. The data show that the two FAs have very different compositions, despite being based on similar starting material and following the same extraction process. ^1H-^{13}C CPSP/MAS NMR experiments were carried out for the simultaneous quantification of mobile and immobile components of soils (Figures S3-S4), as it has been previously shown to be a powerful NMR methodology for the routine analysis of soils in the solid-state. MFA showed a predominant presence of alkyl groups (~75 %), followed by carbonyl (~ 16%), carbohydrate (~ 6%) and methoxyl (~ 3%) components (Figure S3). On the other hand, VFA is mostly composed by carbohydrates (~80%) and a small proportion of alkyl (~ 8%) components; it also contains some carbonyl (~ 7%), phenolic (~ 2%) and aryl (~ 2%) groups (Figure S4). Controlling the inorganic contents of FAs in elemental controls was most feasible (as shown in Supplementary Table S3 and S4), and with the organic contents compensated for with biologically available carbon in the form of sucrose. Controlling where possible these contents were imperative in determining an effect in plant assays and by including nutritional controls we can begin to determine if FAs are acting through a specific pathway with one or two active ingredients or as a nutritional additive. The lack of such controls in previous work may be the reason for the range of responses reported and perhaps for the plant hormone-like stimulatory response after HS application (Canellas and Olivares, 2014; Nardi *et al.*, 1994; Russell *et al.*, 2006; Trevisan *et al.*, 2010a; Trevisan *et al.*, 2010b). Changes such as altered root architecture and uptake may be a nutritional effect. More recently, the standardisation of HS analysis has been advocated, including the separation of C-containing groups (Lamar *et al.*, 2013; Zherebker *et al.*, 2018). Therefore, control solutions for plant and microbial were produced based on elemental analysis for MFA and VFA, termed MC and VC respectively; a description is found in Supplementary Tables S3-S4. Additional controls, as used in other studies, included dH_2O and no application (NA).

Fulvic acid increased growth of *Medicago sativa* and was not a nutritional effect

Biomass yield assays in glasshouse conditions were carried out with *M. sativa* cultivars using applications of MFA and VFA alongside controls solutions MC, VC, NA, and dH_2O. Figure 1 shows vegetative biomass measurements recorded in three independent experiments for cv.

Daisy, Luzelle, and Gea, alongside the grass *L. perenne* cv. AberMagic. Both cultivars Daisy and Luzelle showed significantly increased vegetative growth after 3 weeks of FA treatment when compared to controls; cv. Gea also showed higher growth yields but this increase was not statistically significant. This may be due to more interexperimental variation (as shown in individual sample points) due to the different time period when cv. Gea was tested during colder months in the glasshouse, with more rapid temperature fluctuations. The results in Figure 1 demonstrates how application of FA can increase vegetative yield at very low concentration. This information supports existing indications of a potential yield effect of HS in *M. sativa* and similar forage legumes (Little *et al.*, 2013; Little *et al.*, 2014; Tan and Tantiwiramanond, 1983; Xu *et al.*, 2018). Figure 1 also shows how the grass species *L. perenne* did not have increased vegetative biomass from FA application. Importantly, comparing the nutritional controls to dH$_2$O treatments shows there was no significant nutritional component to the effect of FA application (see Figure 1). Moreover, as shown in Supplementary Figure S5 where FAs are tested with a larger screen of forage species, one can see how vegetative yield increases are found in legume species and not grasses when compared to dH$_2$O only.

Fulvic acid application caused an increased number of pink nodules

Yield in legumes is known to be affected by the degree of root nodulation by symbiosis with *Rhizobium*, including *Sinorhizobium*. The number of nodules were investigated in cultivars Daisy and Luzelle with counts performed on plants grown in both FA and control condition. Figure 2 shows a representative visual scoring of cultivar Daisy nodules for each treatment. This includes labelling of early stage initiating nodules (EIN), established white nodules (WN), or mature pink nodules (PN). Only WN and PN are included in counts.

Total counts are shown in Figure 3, with results from three coded experiments, alongside percentage of pink nodules. In MFA and VFA treatments mean total nodule number was only slightly increased (not significantly), but the number of PN at 21 days was significantly increased compared to all other treatments. The pink colour of large PNs is indicative that *Rhizobium* are actively N-fixing within the nodule, caused by the presence of leghaemoglobin (Liu *et al.*, 2018a). Therefore, FA treatment may affect the rate of N-fixation and thus increase plant vegetative growth. In addition, testing of *M. sativa* on sterile agar plates with FA application alongside control treatments showed that the significant vegetative growth increase was found only when plates were inoculated with *Sinorhizobium meliloti* strain. Plate

experiment phenotypes of vegetative biomass, nodule number, and root biomass of cv. Daisy, showed that increases of the former two are specific to *Rhizobium* inoculation (Supplementary Figure S6).

It is possible that FAs may also directly influence N-fixing bacteria such as *Sinorhizobium*. This has been reported in other studies (Little *et al.*, 2014; Xu *et al.*, 2018), however these papers did not include nutritional controls. Improved symbiotic association of leguminous crops with *Rhizobium* is important with the current emphasis on growing more leguminous crops globally, due to their N-fixing activity (Foyer *et al.*, 2016; Iannetta *et al.*, 2016; Lüscher *et al.*, 2014; Preissel *et al.*, 2015; Reckling *et al.*, 2016). For example, the fixing of atmospheric N_2 in legume/grass pastures has been estimated as $13 - 682$ kg N ha^{-1} yr^{-1} (Ledgard and Steele, 1992). *Medicago sativa* itself has been estimated to fix up to 350 kg N ha^{-1} yr^{-1}, providing an N fixation rate of 0.021 x vegetative dry matter + 16.9 ($R^2 = 0.91$) (Carlsson and Huss-Danell, 2003), regardless of soil status or geographic location. Increased vegetative growth in *Medicago sativa* due to improved symbiosis with *Rhizobium* could have implications for the yields of other cultivated legumes.

Microbial growth is affected by fulvic acid

To determine if FAs affect growth of *Sinorhizobium meliloti* in the absence of plants, microbial growth in liquid culture was tested. Cultures of FAs or controls inoculated with *S. meliloti* were grown over 4 days with cell density tested using the standard microbial techniques of colony forming unit (CFU) counts. The mean cell density results of three independent experiments are shown in Figure 4. No effect of FAs on cell density was found at $0 - 1$ days. At 2 days both FA treated cultures had a higher cell density than their nutritional controls; MFA measured 6.56×10^9 compared to MC at 4.07×10^9; VFA measured 6.81×10^9 compared to VC at 4.26×10^9. By 3 days the MFA treated culture did not differ in its cell density from any controls. Conversely, VFA had a significantly higher cell density of 1.88×10^{10}. These results indicate that adding FAs to liquid cultures can increase growth of *S. meliloti*, with a similar result demonstrated in a study comparing the presence of a HS substance to no addition (Xu *et al.*, 2018). In addition, the effect of FAs on increasing microbial cell growth without the presence of a plant interaction agrees with other published studies; HS addition has been shown to increase growth of *Bradyrhizobium liaoningense* in liquid culture (Guo Gao *et al.*, 2015) and increase general microbial population growth in soil microbial cells (Visser, 1985). In contrast,

a study of *Candida utilis* found no growth change with HS application, so the response may be specific to certain taxa (McLoughlin and Küster, 1972). It is possible that FA is able to affect the growth of other soil microbial populations which may also increase plant vegetative yields. This may include other important *Rhizobium* species for *Medicago* relatives, but moreover species of *Streptomyces*, *Bacillus* and arbuscular mycorrhiza fungi (Schirawski and Perlin, 2018). Comparing the nutritional controls (MC, VC) to distilled water treatments (dH₂O) showed that there was no significant nutritional component to the effect of FA application (see Figure 4).

RNA-seq demonstrates high levels of transcriptional changes in roots following FA treatment after 3 days.

Transcriptional changes were investigated using RNA-seq for *M. sativa* shoot and root tissues treated with either VFA or its nutritional control VC, on the day of treatment (day 0) or three days after the treatment (day 3). Differentially expressed (DE) transcripts were analysed with *de novo* transcriptome assembly, performed to negate for initial bias of other reference alignment such as *M. truncatula* in subsequent analysis. *De novo* transcriptome assembly was successful for building a scaffold, shown in Supplementary Table S7, with similar alignment rates of all transcripts for available *M. truncatula* references (data not shown).

DE transcripts for VFA and VC between the two timepoints was investigated, with transcripts requiring both an absolute log fold change of 0.585 (1.5 x fold change) and a false-discovery rate (FDR) adjusted p-value (q-value) < 0.05; Supplemental Figure S7 shows those between day 0 and day 3 for VFA application in root samples. Figure 5 shows the number of up-regulated (+) and down-regulated (-) DE transcripts in shoots and roots *of M. sativa* following either VFA and/or VC treatment. This result shows that most DE transcripts (1705 upregulated and 241 downregulated) for VFA treatment occurred in the root tissue. This is compared to 140 upregulated and 209 downregulated DE transcripts in the shoot. This study shows that FA as VFA can induce substantial transcriptional changes in *M. sativa* after only three days, with the root showing far higher numbers of DE transcripts than shoots.

Further analysis of DE transcripts is found in Supplemental data, with BLAST results descriptions and analysis of both tissue types, and functional annotation of GO terms and enrichment testing of root samples only. Most DE isoforms had homologues in closely related legume species (Supplementary Figure S8a). Many successful BLAST hits were recorded for

each transcript sequence (Supplementary Figure S8b), with significant hits shown in most having an extremely low E-values close to zero (Supplementary Figure S8c). Following annotation, the GO terms in Supplementary Table S9 and Figure S9 demonstrated that root FA-induced transcriptional changes are wide-ranging for biological process. There are high GO term hits for processes affected by VFA treatment including those regulating transcription and translation, and those associated with oxidation-reduction, metabolism, and transport. The GO analysis provided evidence that VFA very quickly affects crucial pathways in both C and N metabolism, as well as cell wall modification. This rapid transcriptional effect is likely to induce the later yield effect in vegetative tissue. There was indication of changes in responses to defence, stress, and bacteria. These may be linked to response to symbiotic bacteria such as *S. meliloti*; at this developmental stage nodulation can begin to be established and it is well documented that important nodulation genes and factors are associated with defence responses through their evolution and function (Chen *et al.*, 2017; Clúa *et al.*, 2018; Kouchi *et al.*, 2004; Libault *et al.*, 2010; Lohar *et al.*, 2006).

Enrichment testing shown in Supplementary Table S10 further provides evidence that VFA particularly upregulated biological processes associated with N metabolism, alike to findings in Figure S9. Changes in N metabolism in legume species roots is associated with increases in nodulation-signalling during initiation of symbiosis (Liu *et al.*, 2018a). The quick response in transcription in the roots suggests why there is a larger biomass increase after VFA treatment, likely through stimulated N supply to the legume via nodules or uptake by the roots. Responses to bacteria were enriched, providing further evidence from GO analysis that an effect on nodulation initiation may be the cause of such a change. Moreover, other important processes required for new root development and nodule growth showed enrichment, including cell wall biogenesis and organisation. Molecular function testing showed enrichment in root nutrient transporter activity following VFA treatment, as well as enrichment of serine hydrolase activity, which has wide-ranging catalytic activity in plants (Kaschani *et al.*, 2012; Mindrebo *et al.*, 2016).

Transcriptome analysis shows preferential enrichment of nodulation regulation and signalling-related genes

The genes identified from the above analysis in the root which were significantly induced following VFA treatment were noted to overlap considerably with those in studies of early

initiation of nodulation in legumes (Alves-Carvalho *et al.*, 2015; El Yahyaoui *et al.*, 2004; Hayashi *et al.*, 2012; Kant *et al.*, 2016; Kouchi *et al.*, 2004; Larrainzar *et al.*, 2015; Moreau *et al.*, 2011; O'Rourke *et al.*, 2013). To further investigate this DE transcripts were compared to those which have been categorised as specific early symbiotic root nodulation genes in *M. truncatula* by Roux *et al.*, (2014). In this study laser-capture microdissection of roots and nodules was coupled with RNA-seq (Roux *et al.*, 2014). This provided a robust list of genes induced at various stages of nodulation especially in early initiation. Table 1 details those DE transcripts upregulated in the root following VFA treatment which are early genes required for the signalling and regulation of nodulation; annotations are available for many of these. These included an array of transcription factors and domains including *Myb/SANT-like DNA-binding domain protein*, *AP2-like ethylene-responsive transcription factor*, and *zinc finger MYM-type protein 1-like*; AP2/ERF transcription factors are known to control nodule number and differentiation (Middleton *et al.*, 2007; Peng *et al.*, 2017; Vernie *et al.*, 2008; Wang *et al.*, 2010). Many leucine-rich repeat receptor-like kinases (LRR RLKs) and other receptor kinases were found to be highly enriched, for example *LysM domain receptor-like kinase*; many leucine-rich repeat receptor-like kinases including *CLAVATA* protein homologues signal root development and nodulation induction (Krusell *et al.*, 2002; Lim *et al.*, 2011; Mortier *et al.*, 2010; Oka-Kira and Kawaguchi, 2006; Reid *et al.*, 2011; Schnabel *et al.*, 2005), and *LysM-type receptor-like kinases* perceive early *Rhizobium* signals (Amor *et al.*, 2003; Indrasumunar *et al.*, 2011; Kawaharada *et al.*, 2015; Knogge and Scheel, 2006; Limpens *et al.*, 2003; Madsen *et al.*, 2003; Popp and Ott, 2011; Radutoiu *et al.*, 2003; Zipfel and Oldroyd, 2017).

Genes required in bacteria and hormone induced plant responses were found to be upregulated, for example *NDR1/HIN1-like protein 10*, *protein RRP6-like 2*, and *cytokinin hydroxylase-like transcripts*; an increase of *Pathogenesis-related proteins* can be induced in early symbiotic infection, before adequate *Rhizobium* suppression, rather than being in relation to a pathogen response (Clúa *et al.*, 2018; Kouchi *et al.*, 2004; Libault *et al.*, 2010; Lohar *et al.*, 2006; Nakagawa *et al.*, 2011; Oldroyd, 2013; Popp and Ott, 2011). Important chitin regulatory genes are also detected to be changed in their expression by VFA treatment. This may affect lipochitooligosaccharide recognition as the key signal in initiating legume-*Rhizobium* symbiosis (Bozsoki *et al.*, 2017; Dénarié *et al.*, 1996; Liang *et al.*, 2014; Muñoz *et al.*, 2014; Reddy *et al.*, 1998).

Finally, many nodulation specific genes were enriched such as *nodulation-signaling pathway (NSP) proteins*, *NSP-interacting kinases*, and *nodulins*; *nodulin* is crucial in early

nodule development (Becker *et al.*, 2001; Gamas *et al.*, 1998; Kant *et al.*, 2016; Kouchi and Hata, 1993; Legocki and Verma, 1980; Liu *et al.*, 2018b; Marsh *et al.*, 2007; Mathis *et al.*, 1999; Rivers *et al.*, 1997; Roberts and Routray, 2017; Scheres *et al.*, 1990; van de Wiel *et al.*, 1990) including in *M. sativa* (Cheng *et al.*, 2000; Fang and Hirsch, 1998; Lafuente *et al.*, 2010; Pringle and Dickstein, 2004).

The increase in transcription of these genes upon VFA treatment is a strong indication that this HS is associated with inducing early nodulation in *M. sativa*. The effect could be by influencing the plant itself in its response to symbiosis, for example a priming effect of VFA for subsequently inducing infection by the symbiont (Alimadadi *et al.*, 2010; Berg *et al.*, 1989; Harris *et al.*, 2005). Fulvic acid may be able to change the C:N metabolic balance of the plant and thus impact on the regulatory mechanisms of promoting symbiotic nodulation processes (Libault, 2014), or the effect could be a consequence of the treatment on the symbiont causing a nodule number increase. VFA may contain a specific nutritional aid, not adequately controlled for in VC application, which boosts symbiotic *Sinorhizobium* growth in soil and thus makes nodulation happen more rapidly (Singleton and Tavares, 1986; Thies *et al.*, 1991). Or similarly, VFA may decrease the inhibitory role of N in soil on nodulation and thus also encourage nodulation to occur with symbiont and plant (Beauchamp *et al.*, 2001; Zeijl *et al.*, 2018). This is unlikely due to the low N content of the soil used in testing but should be considered.

Vegetative growth effect was recorded in independent field trials

Figure 6 shows a comparison of the vegetative biomass of *Medicago sativa* cultivars in independent field trials following treatment with a fulvic acid or controls, in order to check if the FA treatment effect on *M. sativa* in glasshouse experiments could be demonstrated in the field. Over two years, four trials were conducted at three dedicated forage crop cultivation farms. Trial plots treatments were one of NA, dH$_2$O, VFA, or VC at early establishment of *M. sativa* (April - June). These plots were grown in accordance with site standard management practices for UK forage crop cultivation. Prior to the first harvest of the season (May - July), vegetative biomass was recorded for a sample from each treatment plots, shown in Figure 6 for each experiment. Although different cultivars were tested at the various sites, for each experiment VFA treated plots had increased vegetative biomass. This increased growth compared to NA or VC was 135 – 165 %, which is only slightly lower than measurements from

glasshouse experiments of 167 – 185 %. The biomass increased for all vegetative tissues, both shoot and leaf.

The nutritional content of *M. sativa* tissue from each treatment plot was also assessed for the 2018 trials. The results of one 2018 trial are shown in Supplemental Figure S11 with samples of total vegetative biomass measurements, total chlorophyll, and total protein of samples was recorded. Although there was a significant difference in vegetative biomass, no significant difference in either nutritional content was shown across any treatment. The other 2018 trial had similar results with NA, dH$_2$O, VFA, and VC plots having the average chlorophyll and total protein content as follows; 2.91 and 17.87 %; 2.61 and 17.96 %; 2.83 and 18.21 %; 2.63 and 17.97 %. These measurements show that the yield effect of VFA treatment on *M. sativa* is not linked to changes in tissue nutritional content. The enhanced vegetative growth from nodule stimulation did not result in increased protein storage. It is possible that FA and other HS treatments in other studies may replicate C-containing exudates usually released by plants to aid in symbiosis initiation, which in turn stimulates activity of *Rhizobium*. This increases nodulation signalling which encourages symbiosis, and results of higher nodule activity and thus yield is increased.

It has been suggested that HS have a crucial active ingredient or 'hormone', such as an auxin-like molecule (Canellas *et al.*, 2010; Nardi *et al.*, 1994; Nardi *et al.*, 2002; Russell *et al.*, 2006; Trevisan *et al.*, 2010a; Trevisan *et al.*, 2010b). However, based on the analysis in this project, no such auxin-like molecule was detected and both commercial applications were found to be different from one another. It is possible that past studies lacking nutritional controls may have given auxin-like growth stimulation, as the plants may have been subjected to suboptimal nutritional supply before treatment and growth effects after application could be interpreted as due to a hormonal stimulus. Although FA did promote growth in legumes the response may be complicated by the mixture of many compounds in the product. By performing a chemical fractionation of FAs it may be possible to find several common components with synergistic effects on nodulation.

In conclusion, we have demonstrated a specific stimulatory effect of FA treatment on the early stages of nodulation in *M. sativa* in both the glasshouse and the field. The FA treatment significantly enhanced biomass production and may be relevant for other legume crops.

Acknowledgements

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Tables and Figures

Table 1. Enriched DE transcripts in VFA roots which are putatively classed as highly preferential nodulation regulatory genes and nodule-associated signalling-related genes as in Roux et al., (2014) (Roux et al., 2014); this includes a description of the protein, available gene/protein IDs, the annotation type, and log fold change (logFC) and q-value for each DE transcript.

Gene/Protein ID	Description	Annotation	logFC	q-value
XP_024635034	*Myb/SANT-like DNA-binding domain protein*	TF MYB	5.35	$1.90E^{-06}$
PNX91228	*putative CC-NBS-LRR resistance protein*	LRR	4.98	$1.23E^{-11}$
ABD33274, AES59362, RHN77255	*RALF-like protein*	Calcium/lipid-binding	4.74	$4.63E^{-06}$
RIA81513	*calnexin*	Calcium/lipid-binding	4.67	$6.36E^{-09}$
RHN49201	*wall-associated receptor kinase-like 20*	RLK	4.60	$3.35E^{-05}$
KEH36571, RHN72042	*CLAVATA3/ESR (CLE)-related protein*	Ser/Thr protein kinase	4.36	$2.44E^{-07}$
KEH28705, RHN58556	*putative LRR-domain, L domain-containing protein*	LRR	4.29	$6.43E^{-05}$
XP_003612592, AES95550, RHN54652	*RING-H2 finger protein ATL52-like*	TF ZnFg C2H2	4.28	1.63^{-75}
XP_024641562	*AP2-like ethylene-responsive transcription factor*	TF AP2/ERF	4.08	1.74^{-04}
XP_003594815, AES65066, RHN73104	*COBRA-like protein 7*	COBRA	4.00	$1.78E^{-07}$
XP_003598348, AES68599, RHN65475	*F-box protein interaction domain protein*	F-box protein	3.75	$4.90E^{-07}$
AES76072, AES76110, RHN52304	*NDR1/HIN1-like protein 10*	NHL	3.67	$2.52E^{-04}$
RHN60433	*disease resistance protein (TIR-NBS-LRR class)*	LRR RLK	3.64	$7.66E^{-06}$
XP_013443270, KEH17295, RHN51739	*cytokinin hydroxylase-like*	CK activated	3.60	$6.29E^{-06}$
XP_013466350, KEH40391, RHN77806	*receptor-like protein kinase*	RLK	3.55	$4.72E^{-06}$
XP_003604023, AES74274	*COBRA-like protein 1*	COBRA	3.53	$7.35E^{-07}$

Gene/Protein ID	Description	Annotation	logFC	q-value
RGB31681	*calcium-binding protein*	Calcium/lipid-binding	3.49	$1.84E^{-06}$
RHN72504	*probable inactive receptor kinase At2g26730*	RLK	3.42	$1.32E^{-05}$
XP_003613167, AES96125, RHN55010	*L-tryptophan--pyruvate aminotransferase 1*	TAA1-like	3.39	$2.54E^{-04}$
AES69839	*LRR-P-loop containing nucleoside triphosphate hydrolase*	LRR	3.32	$7.54E^{-06}$
AES91737	*F-box/kelch-repeat protein*	F-box protein	3.32	$6.92E^{-06}$
XP_024637477	*disease resistance protein (TIR-NBS-LRR class)*	LRR	3.24	$1.65E^{-05}$
EXX59026	*WD40 repeat-like protein*	TF WD	3.22	$1.01E^{-04}$
XP_024631685, RHN72543	*mitogen-activated protein kinase kinase kinase 18-like*	STY	3.14	$2.55E^{-05}$
ABD28520	*protein RRP6-like 2*	CK activated	3.09	$9.33E^{-05}$
XP_013451548, KEH25576, RHN50766	*ankyrin repeat/protein kinase domain-containing protein 1*	TF ERF	3.06	$2.97E^{-05}$
AES95938	*disease resistance protein (TIR-NBS-LRR class), putative*	LRR RLK	3.01	$1.91E^{-05}$
RZB96753	*probable LRR receptor-like Ser/Thr-protein kinase*	LRR RLK	2.99	$5.78E^{-05}$
KHN26259	*zinc finger MYM-type protein 1-like*	TF Zn finger	2.95	$1.31E^{-04}$
XP_013451184, KEH25223, RHN50327	*protein NSP-interacting kinase 1*	NSP	2.94	$3.50E^{-05}$
RHN42361	*kinase RLK-Pelle-WAK-LRK10L-1 family*	RLK	2.89	$1.29E^{-04}$
RIA84146	*Ca2+:H+ antiporter*	Calcium/lipid-binding	2.87	$8.02E^{-05}$
AES60803	*F-box plant-like protein*	F-box protein	2.78	$1.71E^{-04}$
XP_013457946, KEH31977, RHN63702	*putative LRR-containing protein*	LRR RLK	2.78	$1.60E^{-04}$
RIA97789	*ARM repeat-containing protein*	E3 ligase	2.72	$2.11E^{-04}$
XP_013445632	*G-type lectin S-receptor-like Ser/Thr-protein kinase*	Ser/Thr protein kinase	2.71	$1.84E^{-04}$
AES73438	*Plant regulator RWP-RK*	NLP	2.70	$1.87E^{-04}$

Gene/Protein ID	Description	Annotation	logFC	q-value
KEH38435	*Rpp4C3*	CK activated	2.69	1.22E^{-04}
RIA81779	*YIF1-domain-containing protein*	TF AP2/ERF	2.69	1.26E^{-04}
AES61923, RHN81250	*C3HC4-type RING zinc finger protein*	TF Zn finger	2.68	1.90E^{-04}
XP_024633471.1	*LysM domain receptor-like kinase 3*	LysM receptor kinase	2.68	1.41E^{-04}
XP_024625794	*putative receptor-like protein kinase*	RLK	2.66	4.17E^{-05}
RHN81081	*proline-rich protein 1-like*	PRP	2.63	5.03E^{-05}
PF04909	*nodulin-6*	NIP	2.56	2.00E^{-04}
XP_003615114, AES98072, RHN56135	*nodulin-26*	NIP	2.56	6.80E^{-05}
XP_013450575, RHN49450	*L-type lectin-domain containing receptor kinase IX.1-like*	RLK	2.49	5.82E^{-05}
XP_013462891, KEH36925, RHN72571	*chitin elicitor receptor kinase 1-like*	LysM receptor kinase	2.48	9.25E^{-06}
XP_003601076.1, AES71327	*nodulation-signaling pathway 2 protein*	NSP	2.11	1.43E^{-05}
XP_024641514, AES76606, RHN52721	*putative NF-X1-type zinc finger protein NFXL1-like protein*	NFX1-type zinc finger	1.75	1.68E^{-05}
XP_013460228, KEH34259, RHN67624	*non-specific phospholipase*	Phospholipase A2	1.52	4.71E^{-06}
XP_024625319	*U-box domain-containing protein 33 isoform X1*	MtPUB	1.37	1.14E^{-04}
XP_003631134, AET05610, RHN43936	*probable inactive receptor kinase At1g48480*	Kinase	1.15	1.75E^{-04}
XP_003616008, AES98966, RHN56723	*CBL-interacting serine/threonine-protein kinase 11*	Calcium binding, Ser/Thr protein kinase	1.04	1.40E^{-04}
RHN48771	*NDR1/HIN1-like protein 1*	NHL	0.88	3.71E^{-05}

Figure legends

Fig. 1. Vegetative biomass of *Medicago* cultivars and *Lolium* following treatment with fulvic acids or controls. Treatments were applied to seedlings at 7 days post germination and vegetative yields (dry weight in mg) were assessed at 21 days post treatment. Treatments were; NA in dark grey; dH$_2$O in grey; 0.5 % MFA in blue; 0.5 % MC in light blue; 1 % VFA in orange; 1 % VC in yellow. Three cultivars of *Medicago* were tested, cv. Daisy (a), Luzelle (b), and Gea (c). One cultivar of *Lolium* was tested, cv. AberMagic (d). Individual seedling biomass was measured for three independent experiments, as shown in black data points (Exp. 1 = circles, Exp. 2 = triangles, Exp. 3 = squares). Box plots show variation across experiments. Multiple comparisons between treatments were conducted using a one-way ANOVA Tukey test shown with letters, and one-tailed student t-tests were calculated for FAs and their controls, with p-value significance indicated left of graphs.

Fig. 2. *Medicago sativa* cv. Daisy nodules following treatment with fulvic acids or controls. Treatments were applied to seedlings at 7 days post germination and photographs above were taken at 21 days post treatment. Treatments were; 1. NA in dark grey; 2. dH$_2$O in grey; 3. 0.5 % MFA in blue; 4. 0.5 % MC in light blue; 5. 1 % VFA in orange; 6. 1 % VC in yellow. Nodules are indicated as either early initiating nodules (EIN), white nodules (WN), or pink nodules (PN). Only white and pink nodules were counted as true nodules in for this analysis. Scale included is 1 mm.

Fig. 3. Nodulation counts of two *Medicago sativa* cultivars following treatment with fulvic acids or controls. Treatments were applied to seedlings at 7 days post germination and nodules counted at 21 days post treatment. Treatments were; NA in dark grey; dH$_2$O in grey; 0.5 % MFA in blue; 0.5 % MC in light blue; 1 % VFA in orange; 1 % VC in yellow. Two cultivars of *Medicago* were tested, cv. Daisy (a), cv. Luzelle (b). Individual seedling nodules were counted for three independent experiments, as

shown in black data points (Exp. 1 = circles, Exp. 2 = triangles, Exp. 3 = squares). Box plots show variation across experiments. Multiple comparisons between treatments were conducted using a one-way ANOVA Tukey test shown with letters, and one-tailed student t-tests were calculated for FAs and their controls, with p-value significance indicated left of graphs.

Fig. 4. Growth effects of fulvic acid on the growth of *Sinorhizobium meliloti* in culture, compared to controls. TY cultures containing treatments as follows were inoculated with *Sinorhizobium meliloti*; NA in dark grey; dH$_2$O in grey; 0.5 % MFA in blue; 0.5 % MC in light blue; 1 % VFA in orange; 1 % VC in yellow. Average colony forming unit (CFU) counts were obtained from triplicate samples on 0 - 4 days of incubation with shaking 220 rpm at 28 °C. Average counts for three separate experiments (3 individual experimental replicates (on separate days), each with 3 technical replicates) were calculated and shown above with standard deviation. Multiple comparisons between treatments were conducted using a one-way ANOVA Tukey test shown with letters.

Fig. 5. Differentially expressed transcripts in *Medicago sativa* shoot and root tissue with treatments of either VFA (orange) or VC (yellow). RNA-seq was carried out on whole shoot and root RNA samples taken on day of treatment (day 0) or three days after treatment (day 3). Transcripts from *de novo* transcriptome assembly with both an absolute log fold change of 0.585 (1.5 x fold change) and a false-discovery rate (FDR) adjusted p-value (q-value) < 0.05 were considered as differentially expressed (DE); DE transcripts significantly expressed between treatments at day 0 were removed to negate for false positives due to experimental variance. The above Venn diagram shows upregulated (+) and downregulated (-) DE transcripts for both treatments between day 0 and day 3, including those which are shared (overlapping region). This difference in DE transcript number is emphasised by differing sizes of the circles in the plot.

Fig. 6. Vegetative biomass of *Medicago sativa* cultivars in independent field trials following treatment with a fulvic acid or controls. Treatments were applied to field plots at beginning of establishment and vegetative yields were assessed before 1st cut of growing season; an area of 625 cm^2 was sampled and total vegetative tissue dried for biomass (in g). Treatments were; no addition (NA in dark grey); deionised water (dH$_2$O in grey); 1 % VFA (VFA in orange); and 1 % VC (VC in yellow). Three trials of four cultivars were run over two years. In 2017 trials were performed at Dengie Crops Ltd. (Southminster, Essex) with four plots per treatment of both cv. Daisy and Fado. In 2018 the trials were at both Blankney Estates Ltd. (Blankney, Lincolnshire) and A Poucher and Sons (Bardney Dairies) Ltd. (Market Rasan, Lincolnshire) with six plots per treatment of cv. Daisy and Gea respectively. Individual plot samples are shown in black data points as indicated, and boxplots are for individual cultivar trials. Relative average increase in yield of VFA treated plots compared to NA is shown as percentage above graph to the nearest 5 %.
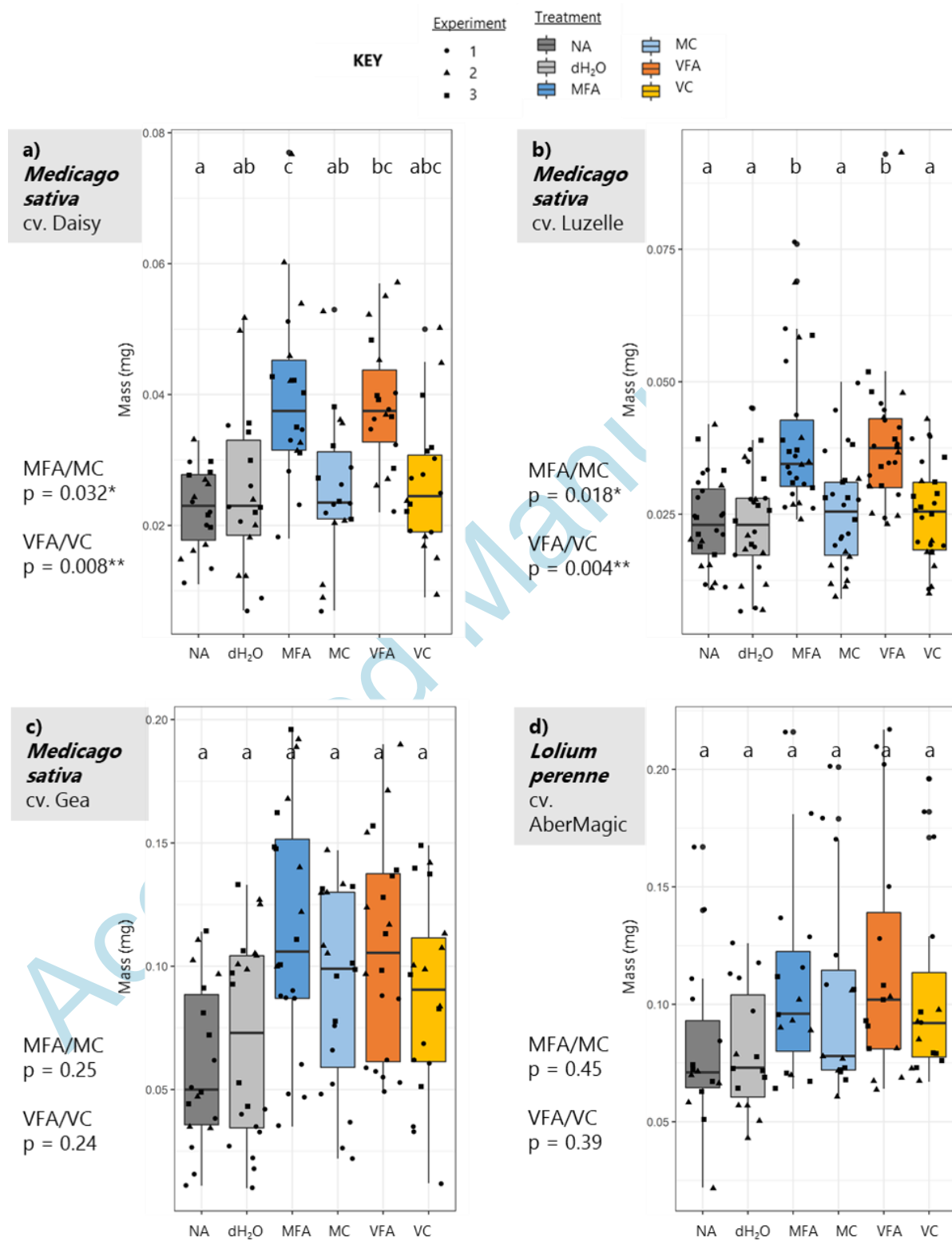
Figure 1

Figure 2

Figure 3

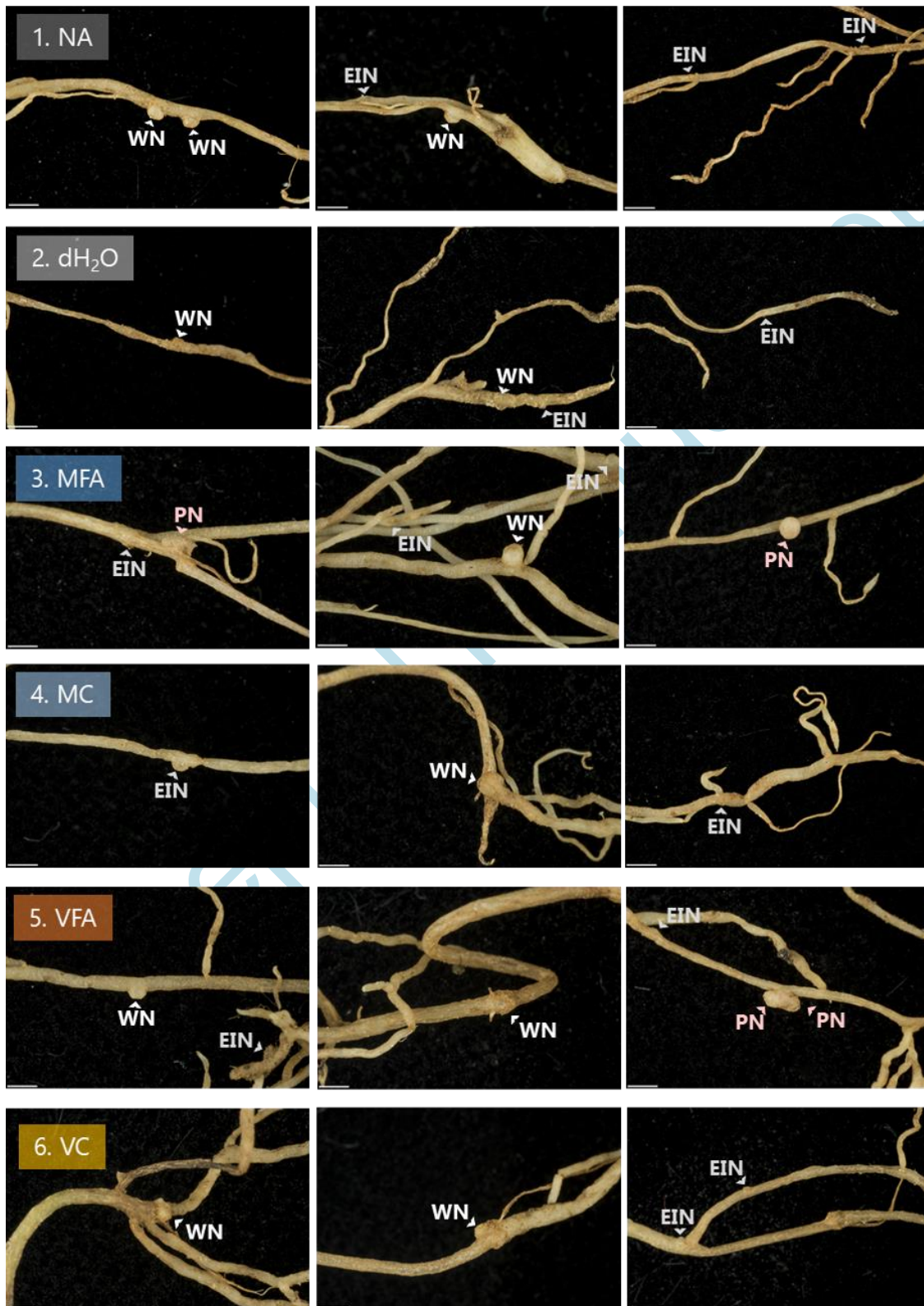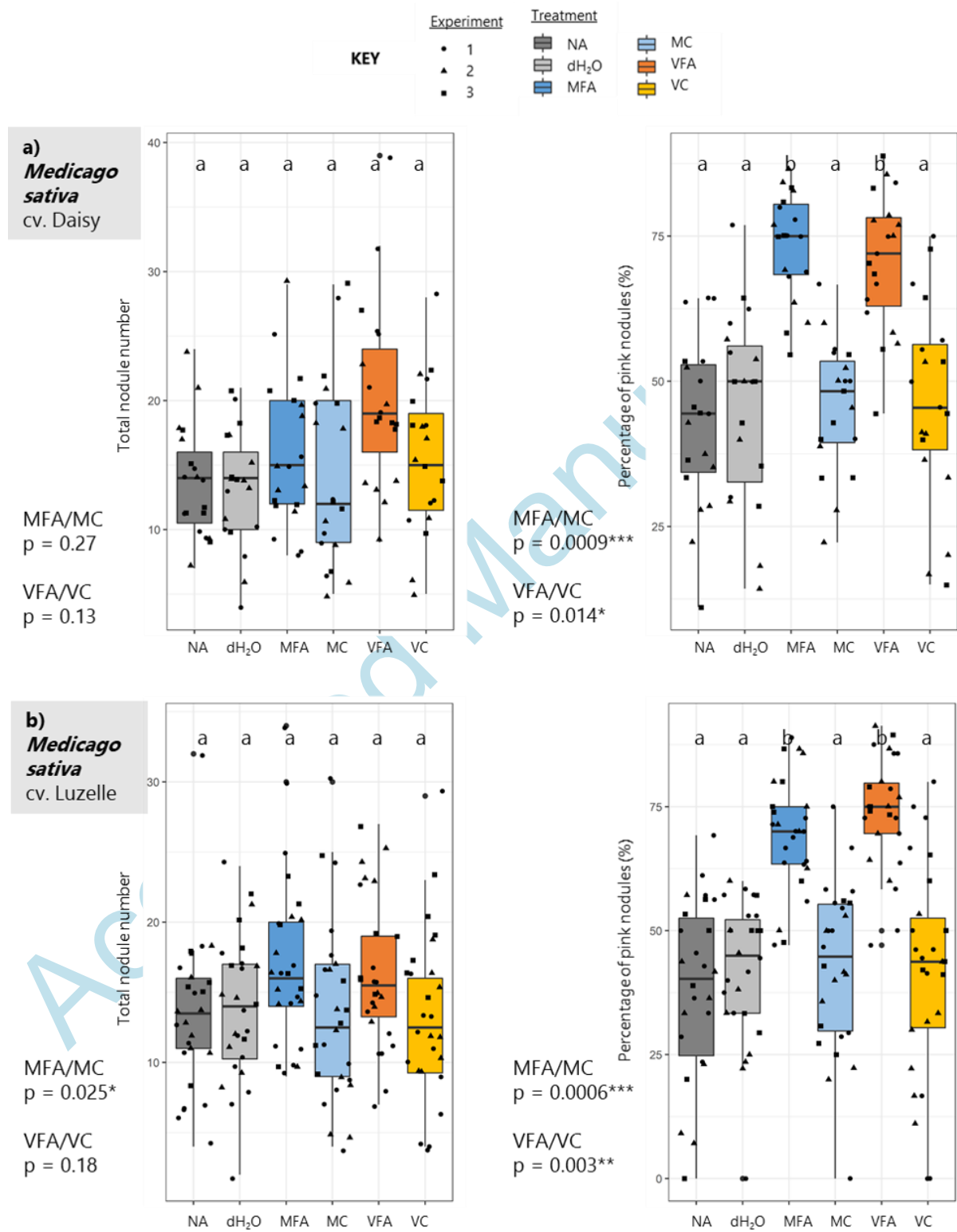a) *Medicago sativa* cv. Daisy

MFA/MC
p = 0.27

VFA/VC
p = 0.13

MFA/MC
p = 0.0009***

VFA/VC
p = 0.014*

b) *Medicago sativa* cv. Luzelle

MFA/MC
p = 0.025*

VFA/VC
p = 0.18

MFA/MC
p = 0.0006***

VFA/VC
p = 0.003**

Figure 4

Figure 5

Figure 6