Metabolomics (2018) 14:143
https://doi.org/10.1007/s11306-018-1440-y

ORIGINAL ARTICLE

NMR-based metabolomics reveals that plant-derived smoke stimulates root growth via affecting carbohydrate and energy metabolism in maize

Şükrü Serter Çatav[1] · Emine Sonay Elgin[2] · Çağdaş Dağ[2,3] · Jaime L. Stark[4] · Köksal Küçükakyüz[1]

Received: 13 June 2018 / Accepted: 5 October 2018 / Published online: 15 October 2018

Abstract

Introduction It is well known that plant-derived smoke stimulates seed germination and seedling growth in many plants. Although a number of transcriptomics and proteomics studies have been carried out to understand the mode of action of smoke, less is known about the biochemical alterations associated with smoke exposure in plants.

Objectives The aims of this study were (1) to determine the metabolic alterations in maize roots pre-treated with various concentrations of smoke solution, and (2) to identify the smoke-responsive metabolic pathways during early root growth period.

Methods Maize seeds were pre-treated with different concentrations of smoke solutions for 24 h and then grown for 10 days. 600-MHz ^{1}H NMR spectroscopy was performed on the aqueous root extracts of maize seedlings. The metabolite data obtained from the NMR spectra were analyzed by several statistical and functional methods, including one-way ANOVA, PCA, PLS-DA and pathway analysis.

Results Our study identified a total of 29 metabolites belonging to various chemical groups. Concentrations of 20 out of these 29 metabolites displayed significant ($p < 0.05$) changes after at least one smoke pre-treatment compared to the control. Moreover, functional analyses revealed that smoke pre-treatments markedly affected the carbohydrate- and energy-related metabolic pathways, such as galactose metabolism, glycolysis, glyoxylate metabolism, tricarboxylic acid cycle, and starch/sucrose metabolism.

Conclusions To our knowledge, this is the first study that investigates smoke-induced biochemical alterations in early root growth period using NMR spectroscopy. Our findings clearly indicate that smoke either directly or indirectly influences many metabolic processes in maize roots.

Keywords Smoke · Root growth · NMR spectroscopy · Metabolomics · Maize

1 Introduction

Fire is one of the most important disturbance factors in terrestrial ecosystems, shaping composition and distribution of plant communities (Bond and Keeley 2005; Keeley et al. 2012). Many plant species have evolved adaptive traits to increase their persistence after fire (Keeley et al. 2011; Nelson et al. 2012). The positive germination response to smoke is one of these traits, and has been demonstrated in numerous plant species from a wide range of phylogenetic origins (Pierce et al. 1995; Adkins and Peters 2001; Moreira et al. 2010; Çatav et al. 2014; Downes et al. 2015). In addition, it has been reported that smoke also triggers root growth (Kulkarni et al. 2006; Van Staden et al. 2006; Wang et al. 2017), somatic embryogenesis (Senaratna et al. 1999; Malabadi and Nataraja 2007), flowering (Keeley 1993; Light

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11306-018-1440-y) contains supplementary material, which is available to authorized users.

✉ Köksal Küçükakyüz
akyuzk@mu.edu.tr

[1] Division of Botany, Department of Biology, Muğla Sıtkı Koçman University, Kötekli, 48000 Muğla, Turkey

[2] Division of Biochemistry, Department of Chemistry, Muğla Sıtkı Koçman University, Kötekli, 48000 Muğla, Turkey

[3] Department of Molecular and Cellular Biochemistry, Indiana University Bloomington, Bloomington, IN 47403, USA

[4] Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA

et al. 2007), and pollen germination (Papenfus et al. 2014) in various plants.

There has been an increasing effort in recent years to understand the mode of action of smoke in germination and early seedling growth processes. Especially, a number of transcriptomics and proteomics studies have been conducted to determine the smoke and smoke-derived compounds [e.g. catechol, karrikinolide (KAR_1) and trimethylbutenolide] related responses in plants, such as *Arabidopsis*, chickpea, lettuce, maize, and wild tobacco (Soós et al. 2009a, 2010, 2012; Nelson et al. 2010; Baldrianová et al. 2015; Wang et al. 2017; Rehman et al. 2018). The results of the transcriptomics analysis of germinating maize seeds indicated that smoke induced the stress and ABA-related responses, promoted the ubiquitination of proteins, and activated the protein-degradation-related genes (Soós et al. 2009a, 2010). Moreover, a proteomics study on the smoke-enhanced root growth in chickpea has revealed that proteins related to amino acid metabolism, starch/sucrose synthesis, glycolysis, tricarboxylic acid (TCA) cycle, nitrate pathway, and secondary metabolism are significantly changed after smoke treatment (Rehman et al. 2018). In addition to these comprehensive approaches, it has been shown that smoke treatments can remarkably alter the contents of protein, total amino acid, total phenolics, photosynthetic pigments, and flavonoids in banana, bean, and *Tulbaghia* species (Aremu et al. 2012, 2014; Singh et al. 2014). However, so far there has been no comprehensive metabolomics evaluation of the effect of smoke on seedling growth.

Metabolomics (or metabonomics), defined as the quantitative measurement of low-molecular-weight metabolites (< 1500 Da) from various biological samples, has great potential to enhance our understanding of biochemical processes in living systems (Fiehn 2002; Hall 2006; Farag et al. 2012; Zhang et al. 2012). The analytical techniques, such as mass spectrometry (MS) coupled to gas and liquid chromatography (GC and LC), and nuclear magnetic resonance (NMR) spectroscopy have been commonly used in metabolomics studies (Lin et al. 2006; Wu et al. 2008; Barding et al. 2013). Identification and quantification of a large number of metabolites from different metabolic classes within a short time frame can be achieved with the help of these powerful techniques (Bino et al. 2004; Zhang et al. 2012).

NMR, although less sensitive than MS, is widely used due to its non-destructive nature and reproducibility (Bino et al. 2004; Pan and Raftery 2007; Emwas 2015). Furthermore, compared with MS-based analyses, the advantages of NMR technique are that it is rapid, and requires a simple sample preparation procedure (Krishnan et al. 2005; Zhi et al. 2012). Additionally, NMR is known to be more effective than GC– and LC–MS analyses in detecting compounds from sugars and amines chemical groups (Wishart 2008; Dai et al. 2010; Barding et al. 2013). Because of the features mentioned above, application of NMR has gained an increasing attention from many fields of research, including biomarker discovery, disease detection, nutritional studies, and plant stress physiology (Odunsi et al. 2005; Gavaghan et al. 2011; Savorani et al. 2013; Zhang et al. 2013).

The stimulatory effect of smoke on root growth of maize (*Zea mays* L.) has been demonstrated in several studies (Sparg et al. 2006; Van Staden et al. 2006; Aslam et al. 2017). Moreover, as stated previously, the transcriptomic profile of germinating maize seeds was significantly altered after smoke treatments (Soós et al. 2009a, 2010). For these reasons, maize was chosen as the experimental material to shed more light on biochemical alterations associated with smoke exposure in plants. In the present study, we performed a seedling growth experiment using maize seeds pre-treated with different concentrations of smoke solution, and employed ^{1}H NMR spectroscopy to assess the metabolite profile of maize root samples. We then utilized functional analysis methods to determine the smoke-responsive metabolic pathways, and discuss their possible roles in root growth process. To the best of our knowledge, this is the first study that examines smoke-induced metabolic changes in root growth process using NMR-based metabolomics.

2 Materials and methods

2.1 Preparation of aqueous smoke solutions

The active compounds of smoke can be generated by subjecting the plant materials or organic compounds (e.g. cellulose, combination of amino acids and sugars) to dry heat (Van Staden et al. 2000; Light et al. 2005; Moreira et al. 2010; Çatav et al. 2014). We used the methodology of Jäger et al. (1996) in order to obtain the active compounds of smoke. Briefly, fresh needles of *Pinus brutia* were collected from the field and broken into small pieces. Three 5-g samples of this plant material were separately heated in closed glass flasks using a temperature-controlled oven at 190 °C for 30 min. Following this process, 50 mL of distilled water was poured over the burnt material and the mixture was allowed to stand for 10 min. Finally, the mixtures were filtered using a Whatman filter paper (no. 1) and solutions obtained (hereafter called smoke solution, and considered as 100%) were placed into a bottle, and stored at 4 °C until used.

2.2 Plant material, growing conditions, and measurement of physical parameters

In this study, maize (genotype: Kompozit Arifiye) seeds were obtained from Institute of Maize Research, Sakarya. Seeds were sterilized with 3% (v/v) sodium hypochlorite and

0.1% (w/v) SDS for 10 min and rinsed with distilled water three times. Surface-sterilized seeds were then incubated in different concentrations of smoke solutions (1%, 2%, 10%, and 100%) for 24 h. Another group of maize seeds were also incubated in distilled water for 24 h and served as the control for the smoke treatments. After the treatments were applied, seeds were directly sown in trays containing perlite. Each treatment consisted of four replicates of 25 seeds. The trays were placed into a growth chamber adjusted to 25 °C with a 16 h photoperiod (at a light intensity of 100 µmol m^{-2} s^{-1}) using a randomized complete block design. Quarter-strength Hoagland's nutrient solution was used to supply essential macro- and micro-nutrients. Seedlings were harvested after 10 days of growth. One part of the fresh root samples was immediately frozen with liquid nitrogen and stored at − 80 °C until freeze-dried within 2 weeks. The photographs of the rest of the seedlings from control and smoke treatments were taken using a digital camera. ImageJ analysis software (version 1.48) was used to measure the root and shoot lengths of each seedling. Furthermore, dry weight of roots and shoots was determined by drying samples in an oven at 70 °C for 48 h.

2.3 Sample preparation for NMR analysis

Approximately, 300 mg fresh root samples were freeze-dried and ground to a fine powder. 650 µL of 0.3 M phosphate buffer solution [in deuterium oxide (D_2O), pH: 7.0] containing 0.5 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS, 97%) was added to the samples. The extracts were sonicated and then centrifuged at 18,000×*g* for 20 min at room temperature. The supernatant of each extract (600 µL) was transferred to NMR tubes for NMR analysis (Gavaghan et al. 2011; Kim et al. 2011).

2.4 NMR spectroscopy

1D ^{1}H NMR spectra were collected at the National Magnetic Resonance Facility at Madison (NMRFAM) on a Bruker Avance III instrument operating at 600 MHz equipped with a triple-resonance cryogenic probe (5 mm) and a SampleJet sample changer. The SampleJet was set at 25 °C, and Topspin 3.2 (Bruker) and NMRbot were used to automate the data collection process (Clos et al. 2013). Moreover, a NOESY-PRESAT pulse sequence was applied to suppress the residual water signal (Kim et al. 2011). Each spectrum consisted of 128 scans of 65,536 complex data points with a spectral width of 9615.4 Hz and acquisition time of 3.41 s. Two spectra for each control sample were collected to evaluate reproducibility and stability. The 1D ^{1}H spectra were phased, baseline corrected, referenced to DSS, and analyzed using the Bruker Topspin 3.2 software.

2.5 Quantification of the metabolite concentrations

Quantification was performed using the 600 MHz library from Chenomx NMR suite professional software (version 7.6), which uses the concentration of a known reference signal (DSS-d6) to assess the concentrations of individual metabolites (Weljie et al. 2006; Kim et al. 2011; Song et al. 2013).

2.6 Data analysis

Growth and metabolite datasets were analyzed by one-way ANOVA followed by Tukey's HSD multiple comparison test. Normality of data and homogeneity of variance were examined using the Shapiro–Wilk's and Bartlett's tests, respectively. When needed, data were subjected to square root or logarithmic transformation in order to meet parametric assumptions.

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) of log-transformed and mean-centered metabolite data were performed using MetaboAnalyst 4.0 software (Xia and Wishart 2016). Moreover, contribution of variables to the principal components (PC) was analyzed using the "FactoMineR" and "factoextra" packages under the R software (3.4.2).

In order to transform the metabolites identified in our experiment into a set of meaningful biological terms (e.g. metabolism-related pathways), we performed two functional analysis using MBRole and MetaboAnalyst online analysis tools. Firstly, an overrepresentation (enrichment) analysis of metabolites, which were significantly affected after at least one smoke treatment, was performed with using pathway database of maize (KEGG) in MBrole 2.0. In this analysis, *p* values were calculated with the cumulative hypergeometric distribution function, and then adjusted for multiple testing using the false discovery rate (FDR) method (López-Ibáñez et al. 2016). Secondly, a quantitative pathway analysis was conducted for control and smoke pre-treatment (10%) datasets in MetaboAnalyst 4.0. We used the *Oryza sativa* (rice) metabolic pathway database (KEGG) as a reference for 'global test' and 'relative betweenness centrality' algorithms (Xia and Wishart 2016).

For all statistical analyses, the significance level was set at 0.05.

3 Results

3.1 Effect of smoke pre-treatments on seedling growth

Effect of pre-treatment of maize seeds with various concentrations of smoke solution on root and shoot growth is shown

in Fig. 1. Root length and root dry weight of seedlings pre-treated with 10% and 100% smoke solutions were markedly ($p < 0.05$) increased in comparison with the control (Fig. 1a, b). Furthermore, smoke pre-treatment at the concentration of 10% resulted in a significant enhancement in the number of seminal roots (Fig. 1e). On the other hand, none of the smoke pre-treatments remarkably affected the shoot length and shoot dry weight (Fig. 1c, d). Our results also indicated that root/shoot ratio increased almost linearly with the increasing concentration of smoke solution (Fig. 1f).

3.2 Identification and quantification of NMR data

Comparison of representative ^{1}H NMR spectra (600 MHz) obtained from extracts of control and smoke pre-treatment (10%) root samples is shown in Fig. 2. A total of 29 metabolites, which belonged to various chemical groups such as alcohols, amino acids, organic acid derivatives, and sugars, were identified in these NMR spectra (Table 1). While sugar peaks were primarily observed between 3.00 and 5.50 ppm, most of the amino acids peaks were seen in the aliphatic region (about 0.50–3.00 ppm).

Of the 29 metabolites quantified, 20 showed significant ($p < 0.05$) changes in concentration after at least one smoke pre-treatment compared to control (Table 1). Smoke pre-treatments led to significant increases in the levels of metabolites such as alanine, isoleucine, leucine, valine, glucose, fructose, and methanol in root tissue of maize seedlings. On the other hand, the levels of *N*-acetyltyrosine, sarcosine, sucrose, galactitol, lactate, and ethanol were significantly decreased in most of the smoke pre-treatment samples (Table 1).

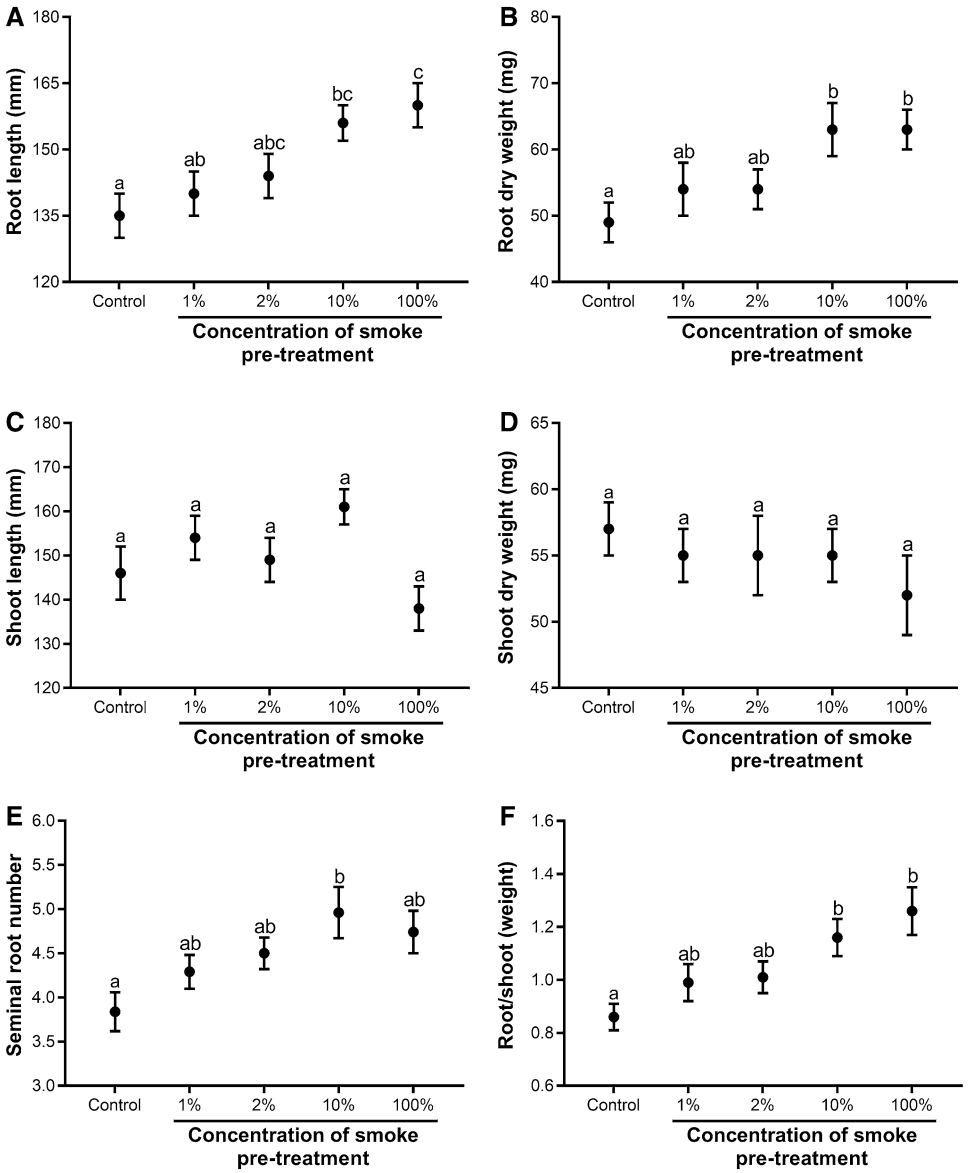

Fig. 1 Effect of smoke pre-treatments on **a** root length, **b** root dry weight, **c** shoot length, **d** shoot dry weight, **e** seminal root number, and **f** root/shoot ratio. Different letters on the error bars indicate significant differences ($p < 0.05$) according to Tukey's HSD test. Results are presented as mean (n = 4 replicates of 18 samples per treatment) ± standard error

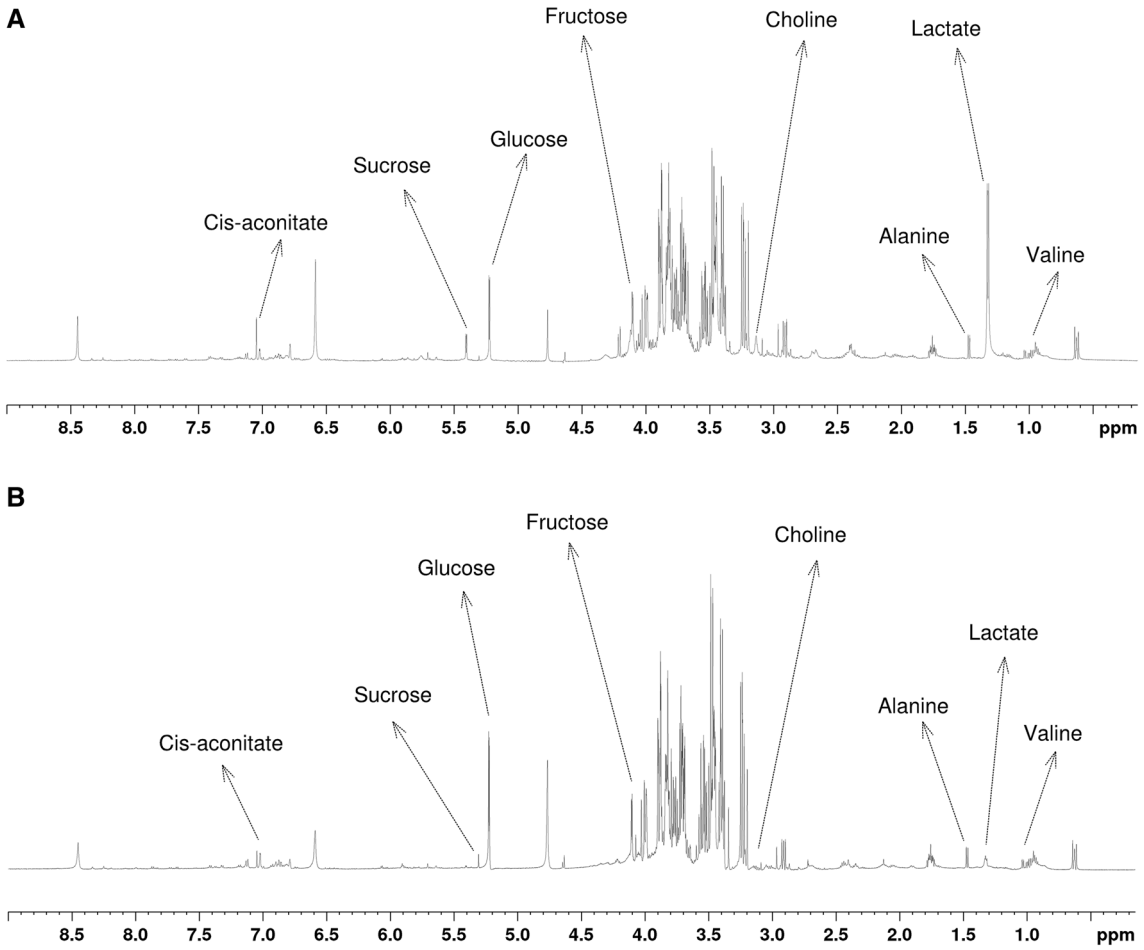

Fig. 2 Comparative 1D ^{1}H NMR spectra obtained from extracts of **a** control and **b** smoke pre-treated (10%) root samples

3.3 Multivariate analyses of metabolite data

In the PCA analysis, the first three components, which had eigenvalues greater than 1, accounted for 37.5%, 26.9%, and 14.5% of the total variation in the metabolite data, respectively. The PCA scores plot in Fig. 3a shows that 10% and 100% smoke pre-treatments, which significantly enhance root growth, are closely clustered, while control and rest of the smoke pre-treatments (1% and 2%) are separated. Moreover, the PCA loadings plot indicates that lactate, methanol, sucrose, sarcosine, galactitol, *N*-acetyltyrosine, and isoleucine are the main metabolites responsible for separation between the groups (Fig. 3b).

The scores plot of PLS-DA (Fig. 4a) reveals a clear separation between the groups suggesting that metabolite changes are unique to each smoke pre-treatment ($R^2 = 0.960$, $Q^2 = 0.842$, $p < 0.001$ by permutation test). Furthermore, the variables important in projection (VIP) scores plot in Fig. 4b demonstrates that lactate, methanol, galactitol, sarcosine, glucose, isoleucine, and *N*-acetyltyrosine are the most important metabolites in group differentiation.

3.4 Smoke-responsive metabolic pathways

The common results of overrepresentation and pathway analyses showed that smoke pre-treatments markedly affected the galactose metabolism, glycolysis/gluconeogenesis, starch/sucrose metabolism, and branched-chain amino acid (BCAA) biosynthesis in maize roots (Table 2; Fig. 5, Table S1). The pathway analysis results also suggested that pyruvate metabolism, glyoxylate/dicarboxylate metabolism, glycerolipid metabolism, and TCA cycle might be significantly influenced by smoke pre-treatments (Fig. 5, Table S1).

4 Discussion

It is well recognized that smoke and smoke-derived compounds stimulate both root and shoot growth in many plants from different ecological and taxonomic groups (Kulkarni et al. 2006; Van Staden et al. 2006; Moreira et al. 2010; Yearsley et al. 2018). It has also been shown that smoke is

Table 1 Concentrations (mM) of the identified metabolites in the control and in the smoke treatments

Metabolite	CG	Control	Concentration of smoke pre-treatment (%)			
			1	2	10	100
Ethanol	ALC	0.024 ± 0.003^{c}	0.014 ± 0.002^{ab}	0.018 ± 0.001^{ac}	0.023 ± 0.003^{bc}	0.012 ± 0.001^{a}
Methanol	ALC	0.027 ± 0.003^{a}	0.050 ± 0.006^{a}	0.037 ± 0.001^{a}	0.133 ± 0.018^{b}	0.113 ± 0.019^{b}
1-Methylhistidine	A&A	0.023 ± 0.003^{a}	0.027 ± 0.003^{ab}	0.033 ± 0.003^{ab}	0.050 ± 0.010^{b}	0.024 ± 0.000^{a}
Alanine	A&A	0.059 ± 0.011^{a}	0.102 ± 0.001^{b}	0.098 ± 0.003^{b}	0.097 ± 0.007^{ab}	0.083 ± 0.013^{ab}
Dimethylglycine	A&A	0.002 ± 0.000^{a}	0.003 ± 0.000^{a}	0.006 ± 0.001^{b}	0.003 ± 0.000^{a}	0.004 ± 0.000^{ab}
Glycine	A&A	0.182 ± 0.004^{a}	0.218 ± 0.012^{a}	0.218 ± 0.014^{a}	0.213 ± 0.013^{a}	0.218 ± 0.015^{a}
Histamine	A&A	0.022 ± 0.002^{a}	0.013 ± 0.003^{a}	0.013 ± 0.001^{a}	0.020 ± 0.000^{a}	0.013 ± 0.002^{a}
Isoleucine	A&A	0.019 ± 0.003^{a}	0.035 ± 0.004^{ab}	0.054 ± 0.003^{b}	0.050 ± 0.003^{b}	0.047 ± 0.007^{b}
Leucine	A&A	0.035 ± 0.005^{a}	0.062 ± 0.005^{b}	0.091 ± 0.005^{c}	0.047 ± 0.003^{ab}	0.060 ± 0.006^{b}
N-Acetyltyrosine	A&A	0.038 ± 0.004^{b}	0.028 ± 0.003^{ab}	0.017 ± 0.002^{a}	0.024 ± 0.003^{a}	0.020 ± 0.001^{a}
Phenylalanine	A&A	0.028 ± 0.002^{a}	0.031 ± 0.001^{a}	0.031 ± 0.002^{a}	0.030 ± 0.001^{a}	0.024 ± 0.002^{a}
Putrescine	A&A	0.061 ± 0.009^{a}	0.050 ± 0.001^{a}	0.045 ± 0.002^{a}	0.043 ± 0.007^{a}	0.041 ± 0.003^{a}
Pyroglutamate	A&A	0.131 ± 0.012^{a}	0.133 ± 0.012^{a}	0.084 ± 0.007^{a}	0.126 ± 0.007^{a}	0.123 ± 0.030^{a}
Sarcosine	A&A	0.012 ± 0.001^{b}	0.006 ± 0.001^{a}	0.003 ± 0.000^{a}	0.004 ± 0.000^{a}	0.005 ± 0.000^{a}
Tyrosine	A&A	0.030 ± 0.004^{a}	0.044 ± 0.003^{a}	0.046 ± 0.003^{a}	0.025 ± 0.003^{a}	0.045 ± 0.009^{a}
Valine	A&A	0.027 ± 0.004^{a}	0.039 ± 0.004^{ab}	0.050 ± 0.002^{b}	0.050 ± 0.003^{b}	0.054 ± 0.005^{b}
2-Hydroxy-phenylacetate	OAD	0.035 ± 0.004^{ab}	0.033 ± 0.003^{ab}	0.032 ± 0.002^{ab}	0.047 ± 0.006^{b}	0.020 ± 0.001^{a}
3-Methylglutarate	OAD	0.025 ± 0.004^{a}	0.040 ± 0.006^{ac}	0.048 ± 0.003^{bc}	0.060 ± 0.006^{c}	0.034 ± 0.003^{ab}
Cis-aconitate	OAD	0.016 ± 0.001^{a}	0.021 ± 0.001^{ab}	0.043 ± 0.003^{d}	0.035 ± 0.001^{cd}	0.027 ± 0.003^{bc}
Lactate	OAD	0.547 ± 0.007^{b}	0.660 ± 0.061^{b}	0.860 ± 0.032^{c}	0.303 ± 0.032^{a}	0.153 ± 0.006^{a}
Malonate	OAD	0.090 ± 0.006^{b}	0.047 ± 0.003^{a}	0.153 ± 0.003^{c}	0.065 ± 0.005^{ab}	0.087 ± 0.009^{b}
Pyruvate	OAD	0.020 ± 0.001^{a}	0.025 ± 0.004^{a}	0.025 ± 0.002^{a}	0.027 ± 0.002^{a}	0.021 ± 0.003^{a}
Fructose	S&SA	0.917 ± 0.101^{a}	1.110 ± 0.078^{ab}	1.590 ± 0.076^{bc}	1.677 ± 0.148^{c}	1.557 ± 0.147^{bc}
Galactitol	S&SA	0.189 ± 0.016^{cd}	0.213 ± 0.015^{d}	0.103 ± 0.009^{ab}	0.140 ± 0.010^{bc}	0.073 ± 0.012^{a}
Glucose	S&SA	2.960 ± 0.091^{a}	2.977 ± 0.133^{a}	4.450 ± 0.266^{b}	6.650 ± 0.029^{c}	6.083 ± 0.060^{c}
Glycerol	S&SA	0.096 ± 0.008^{a}	0.130 ± 0.006^{a}	0.127 ± 0.015^{a}	0.173 ± 0.003^{b}	0.123 ± 0.009^{a}
Sucrose	S&SA	0.360 ± 0.010^{c}	0.163 ± 0.009^{ab}	0.120 ± 0.010^{a}	0.257 ± 0.037^{b}	0.440 ± 0.021^{c}
Choline	Others	0.090 ± 0.010^{a}	0.107 ± 0.003^{a}	0.107 ± 0.010^{a}	0.083 ± 0.009^{a}	0.087 ± 0.011^{a}
Xanthosine	Others	0.007 ± 0.001^{a}	0.011 ± 0.001^{a}	0.011 ± 0.001^{a}	0.019 ± 0.001^{b}	0.011 ± 0.001^{a}

Results are presented as mean (n = 3) ± standard error. Values with different superscript letters in the same raw are significantly different ($p < 0.05$, Tukey's HSD test)

CG chemical group (*ALC* alcohols, *A&A* amino acids and amines, *OAD* organic acid derivatives, *S&SA* sugar and sugar alcohols)

more effective in enhancing root growth relative to shoot growth (Brown and Van Staden 1997; Moreira et al. 2010; Wang et al. 2017; Çatav et al. 2018). The results of our study are consistent with those findings, and indicate that smoke pre-treatments significantly stimulate the primary and seminal root growth in maize in a concentration-dependent manner (Fig. 1). This is very important because primary and seminal roots play a key role in water and nutrient uptake, especially in early seedling stages (Zhu et al. 2006; Singh et al. 2010). Therefore, our findings suggest that smoke pre-treatments may provide an advantage to seedling establishment rate in maize.

Our study identified a total of 14 metabolites belonging to amino acid and amine groups in maize root samples. Concentrations of 8 out of these 14 metabolites displayed significant ($p < 0.05$) changes after smoke pre-treatments (Table 1). Especially, the levels of amino acids, such as alanine, isoleucine, leucine, and valine, in root samples were markedly increased in most of the smoke pre-treatments. Moreover, functional analyses revealed that smoke pre-treatments remarkably affected the valine, leucine, and isoleucine biosynthesis in maize roots (Table 2; Fig. 5, Table S1). Our findings support previous studies showing that smoke treatments affect the amino acid metabolism, and change the free amino acid content (Singh et al. 2014; Rehman et al. 2018). In addition, an increase in the free amino acid content may partially be explained by enhanced protein degradation (Chyliński et al. 2007). It has been suggested that in germinating maize seeds, smoke

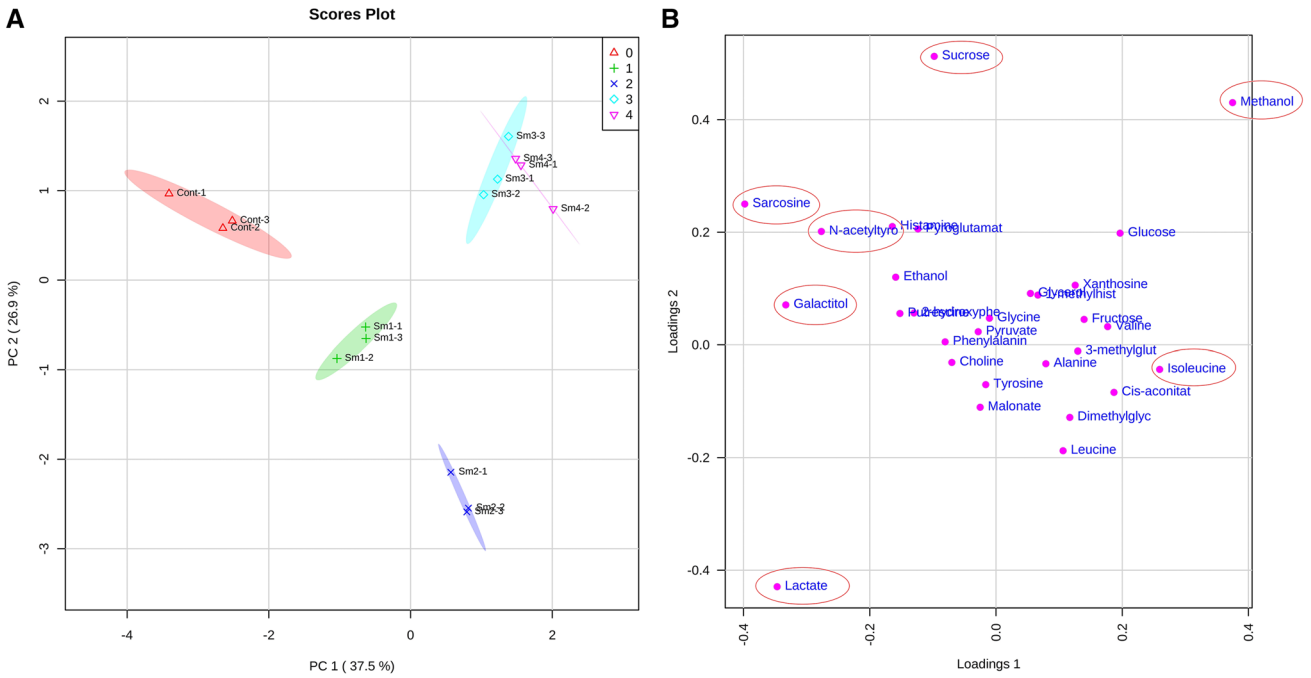

Fig. 3 **a** PCA scores plot obtained from the metabolite data of maize root samples subjected to different smoke pre-treatments. **b** PCA loadings plot for the selected PCs. The circles indicate the most important metabolites responsible for separation of treatment groups. The abbreviations represent the concentrations of the smoke pre-treatments (Sm1 = 1%, Sm2 = 2%, Sm3 = 10%, and Sm4 = 100%)

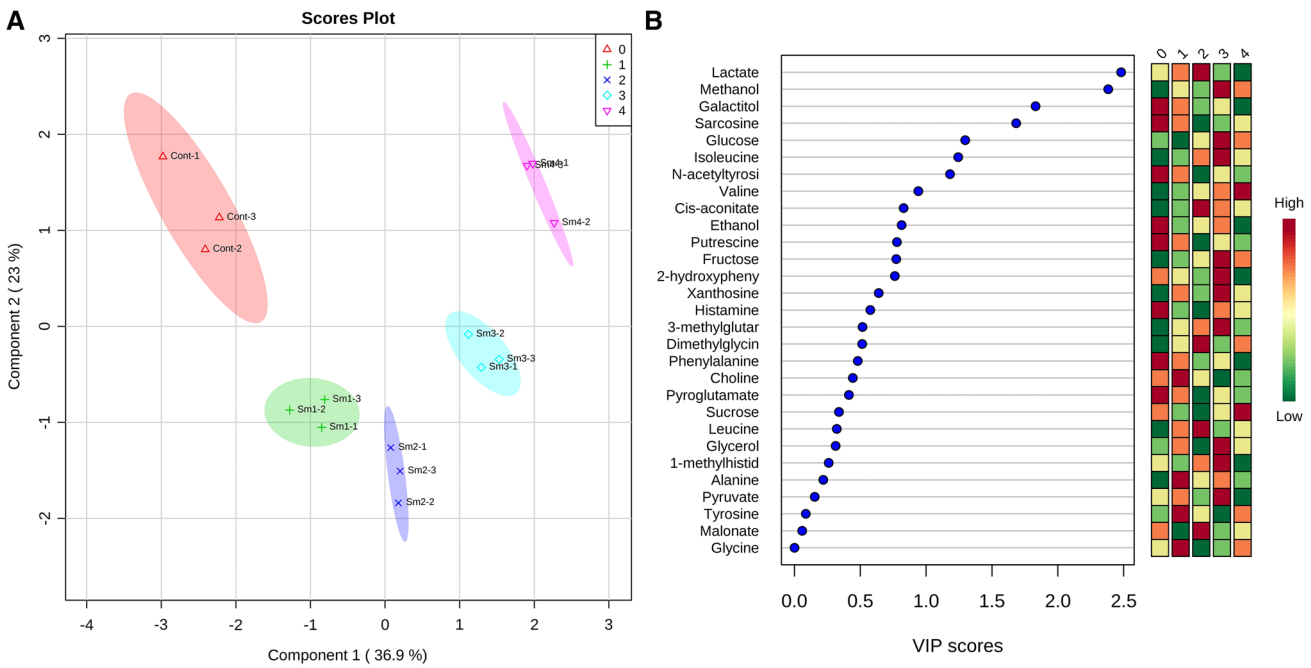

Fig. 4 **a** PLS-DA scores plot obtained from the metabolite data of maize root samples subjected to different smoke pre-treatments. **b** The most important metabolites responsible for the group separation ranked by the PLS-DA VIP (variable importance in projection) scores. The heat map on the right shows the relative concentrations of the corresponding metabolite in each treatment group. The abbreviations represent the concentrations of the smoke pre-treatments (Sm1 = 1%, Sm2 = 2%, Sm3 = 10%, and Sm4 = 100%)

Table 2 Summary of the smoke-responsive metabolic pathways obtained from MBRole 2.0 by overrepresentation (enrichment) analysis

Pathway name	KEGG pathway code	Total	Hits	Statistics	
				p Value	FDR correction
Galactose metabolism	zma00052	18	5	< 0.0001	< 0.0001
Metabolic pathways	zma01100	18	15	0.0007	0.0043
Aminoacyl-tRNA biosynthesis	zma00970	18	4	0.0006	0.0043
Glycolysis/gluconeogenesis	zma00010	18	3	0.0005	0.0043
Valine, leucine and isoleucine biosynthesis	zma00290	18	3	0.0004	0.0043
Valine, leucine and isoleucine degradation	zma00280	18	3	0.0013	0.0067
Starch and sucrose metabolism	zma00500	18	3	0.0022	0.010
Biosynthesis of secondary metabolites	zma01110	18	8	0.167	0.235

In the table, "total" is the total number of metabolites in the relevant pathway; "hits" is the actually matched number of metabolites from the uploaded data set. For more details, see Sect. 2.6

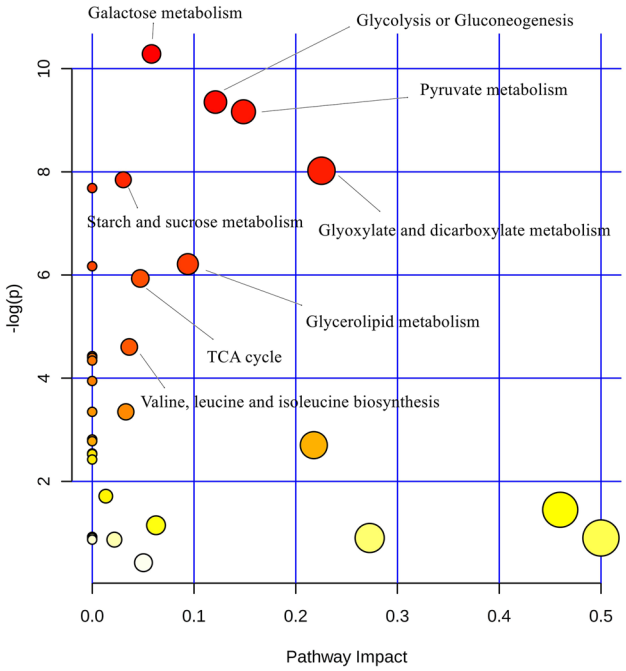

Fig. 5 Schematic presentation of smoke-responsive metabolic pathways obtained from quantitative pathway analysis. For details, see Table S1

also promotes the ubiquitination of proteins, and activates protein-degradation-related genes (Soós et al. 2010).

Soluble sugars (e.g. fructose, glucose, and sucrose) play a major role in regulating many processes in plants, including growth, development, and stress tolerance (Smeekens and Rook 1997; Couée et al. 2006; Rolland et al. 2006; Rosa et al. 2009). Moreover, sucrose-cleaving enzymes, such as invertase and sucrose synthase are known to have pivotal functions in these regulatory mechanisms (Sturm and Tang 1999; Koch 2004). For instance, it has been suggested that invertase-mediated glucose signaling can induce the expression of genes associated with cell cycle, cell division, and auxin biosynthesis, which directly influence the growth rate in plants (LeClere et al. 2010; Ruan 2012). Our results reveal that the levels of glucose and fructose show an upward trend in response to smoke pre-treatments, while that of sucrose shows a relative declining trend, except for 100% concentration of smoke pre-treatment (Table 1). This finding suggests that conversion of sucrose to hexoses may be promoted by smoke in early root growth period. Furthermore, both pathway and overrepresentation analyses indicated that smoke pre-treatments significantly influenced the sucrose metabolism in maize roots (Table 2; Fig. 5, Table S1). A similar result was also obtained by Rehman et al. (2018), who found that smoke treatment triggered the sucrose synthase activity, and increased the total soluble sugar content. In the light of these data, it seems possible that smoke may affect the activity of sucrose-cleaving enzymes that have important roles in regulating plant growth and development. In addition to these, functional analysis demonstrated that smoke pre-treatments had the highest impact on galactose metabolism (Table 2; Fig. 5). Sugar alcohols, such as galactitol and glycerol are substantial components of this metabolism, and their concentrations were remarkably changed by smoke pre-treatments. Taken as a whole, our results provide evidence that changes in sucrose and galactose metabolisms take an important place in smoke-stimulated root growth in maize.

Gene expression and proteomics analyses revealed that metabolic pathways such as glycolysis and TCA cycle may be affected by smoke and KAR_1. For instance, Soós et al. (2009b) demonstrated that smoke led to induction in the expression of pyruvate kinase, a key enzyme of glycolysis, in germinating lettuce seeds. Moreover, a significant effect of KAR_1 on TCA cycle-related proteins was detected in *Arabidopsis* seedlings (Baldrianová et al. 2015). Additionally, Rehman et al. (2018) reported that in chickpea roots, the abundance of proteins, which were related to glycolysis

and TCA cycle, was markedly altered in response to smoke treatment. Our results also support these findings by showing that glycolysis and TCA cycle were responsive to smoke pre-treatments in metabolite level (Fig. 5).

Our pathway analysis results suggest that glyoxylate and dicarboxylate metabolism was also affected by smoke (Fig. 5, Table S1). The glyoxylate cycle, an anaplerotic pathway, allows bypassing the two decarboxylation steps of the TCA cycle, resulting in the conversion of two-carbon acetyl units into four-carbon units (succinate) for energy production (Eastmond et al. 2000; Berg et al. 2002). In many seeds, this cycle provides energy for germination and seedling growth via conversion of storage triacylglycerols to sugars (Eastmond and Graham 2001; Cornah and Smith 2002). Therefore, changes observed in the levels of soluble sugars might also be associated with induction of this pathway by smoke (Table 1). To sum up, on the basis of the results of our study and the previous research mentioned above, it seems reasonable to conclude that plant-derived smoke can influence the various energy-related pathways during seedling establishment and growth.

One interesting finding of this study was that smoke pre-treatments at concentrations of 10% and 100%, which stimulated root growth, resulted in a significant increase in methanol content of maize roots (Table 1; Fig. 1). It has been demonstrated that methanol accumulation in plants is highly correlated with increasing activity of pectin methylesterase (PME), a cell-wall-bound enzyme (Micheli 2001; Komarova et al. 2014; Kohli et al. 2015). Moreover, PME-mediated changes in cell wall are known to play a crucial role in diverse physiological processes, including seed germination, root elongation, and fruit ripening (Pelloux et al. 2007; Wolf et al. 2009). In view of these arguments, it can be proposed that PME activity may be affected by smoke pre-treatments in a concentration-dependent manner. However, further evidence-based studies are required to verify this assumption.

5 Conclusion

The results of present study indicate that smoke pre-treatments (1) stimulate root growth in maize in a concentration-dependent manner, (2) significantly alter the levels of most of the identified metabolites in maize roots, and (3) markedly affect the carbohydrate- and energy-related metabolic pathways. Furthermore, multivariate analyses suggest that metabolites such as lactate, methanol, sarcosine, galactitol, isoleucine, and *N*-acetyltyrosine can be potential biomarkers for smoke-stimulated root growth. In conclusion, we strongly believe that further studies using a combined omics approach are required to improve our understanding of the mechanisms underlying smoke-stimulated seedling growth.

Acknowledgements This work is a part of Ph.D. thesis of the first author, funded by the Scientific Research Projects Coordination Unit of Muğla Sıtkı Koçman University (Grant Numbers: 13/02 and 15/153). This study made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH Grant P41GM103399 (NIGMS), old number: P41RR002301. Equipment was purchased with funds from the University of Wisconsin-Madison, the NIH (P41GM103399, S10RR02781, S10RR08438, S10RR023438, S10RR025062, S10RR029220), the NSF (DMB-8415048, OIA-9977486, BIR-9214394), and the USDA.

Author contributions ŞSÇ, ÇD, ESE and KK designed the experiments, ŞSÇ and ÇD performed the experiments, JLS and ÇD carried out the acquisition of the NMR data, ŞSÇ and ESE analyzed the data and wrote the first draft. All authors critically revised, read, and approved the final version of manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Adkins, S. W., & Peters, N. C. B. (2001). Smoke derived from burnt vegetation stimulates germination of arable weeds. *Seed Science Research, 11*(3), 213–222.

Aremu, A. O., Bairu, M. W., Finnie, J. F., & Van Staden, J. (2012). Stimulatory role of smoke–water and karrikinolide on the photosynthetic pigment and phenolic contents of micropropagated 'Williams' bananas. *Plant Growth Regulation, 67*(3), 271–279.

Aremu, A. O., Masondo, N. A., & Van Staden, J. (2014). Smoke–water stimulates secondary metabolites during in vitro seedling development in *Tulbaghia* species. *South African Journal of Botany, 91*, 49–52.

Aslam, M. M., Jamil, M., Khatoon, A. A., Hendawy, S. E., Al-Suhaibani, N. A., Malook, I., et al. (2017). Physiological and biochemical responses of maize (*Zea mays* L.) to plant derived smoke solution. *Pakistan Journal of Botany, 49*(2), 435–443.

Baldrianová, J., Černỳ, M., Novák, J., Jedelskỳ, P. L., Divíšková, E., & Brzobohatỳ, B. (2015). *Arabidopsis* proteome responses to the smoke-derived growth regulator karrikin. *Journal of Proteomics, 120*, 7–20.

Barding, G. A. Jr., Béni, S., Fukao, T., Bailey-Serres, J., & Larive, C. K. (2013). Comparison of GC-MS and NMR for metabolite profiling of rice subjected to submergence stress. *Journal of Proteome Research, 12*(2), 898–909.

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). *Biochemistry* (5th ed.). New York: WH Freeman and Company.

Bino, R. J., Hall, R. D., Fiehn, O., Kopka, J., Saito, K., Draper, J., et al. (2004). Potential of metabolomics as a functional genomics tool. *Trends in Plant Science, 9*(9), 418–425.

Bond, W. J., & Keeley, J. E. (2005). Fire as a global 'herbivore': The ecology and evolution of flammable ecosystems. *Trends in Ecology & Evolution, 20*(7), 387–394.

Brown, N. A. C., & Van Staden, J. (1997). Smoke as a germination cue: A review. *Plant Growth Regulation, 22*(2), 115–124.

Çatav, ŞS., Küçükakyüz, K., Akbaş, K., & Tavşanoğlu, Ç (2014). Smoke-enhanced seed germination in Mediterranean Lamiaceae. *Seed Science Research, 24*(3), 257–264.

Çatav, ŞS., Küçükakyüz, K., Tavşanoğlu, Ç, & Pausas, J. G. (2018). Effect of fire-derived chemicals on germination and seedling growth in Mediterranean plant species. *Basic and Applied Ecology, 30*, 65–75.

Chyliński, W. K., Lukaszewska, A. J., & Kutnik, K. (2007). Drought response of two bedding plants. *Acta Physiologiae Plantarum, 29*(5), 399.

Clos, L. J., Jofre, M. F., Ellinger, J. J., Westler, W. M., & Markley, J. L. (2013). NMRbot: Python scripts enable high-throughput data collection on current Bruker BioSpin NMR spectrometers. *Metabolomics, 9*(3), 558–563.

Cornah, J. E., & Smith, S. M. (2002). Synthesis and function of glyoxylate cycle enzymes. In *Plant peroxisomes* (pp. 57–101). Dordrecht: Springer.

Couée, I., Sulmon, C., Gouesbet, G., & El Amrani, A. (2006). Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany, 57*(3), 449–459.

Dai, H., Xiao, C., Liu, H., Hao, F., & Tang, H. (2010). Combined NMR and LC–DAD–MS analysis reveals comprehensive metabonomic variations for three phenotypic cultivars of *Salvia miltiorrhiza* Bunge. *Journal of Proteome Research, 9*(3), 1565–1578.

Downes, K. S., Light, M. E., Pošta, M., & van Staden, J. (2015). Fire-related cues and the germination of eight *Conostylis* (Haemodoraceae) taxa, when freshly collected, after burial and after laboratory storage. *Seed Science Research, 25*(3), 286–298.

Eastmond, P. J., Germain, V., Lange, P. R., Bryce, J. H., Smith, S. M., & Graham, I. A. (2000). Postgerminative growth and lipid catabolism in oilseeds lacking the glyoxylate cycle. *Proceedings of the National Academy of Sciences, 97*(10), 5669–5674.

Eastmond, P. J., & Graham, I. A. (2001). Re-examining the role of the glyoxylate cycle in oilseeds. *Trends in Plant Science, 6*(2), 72–78.

Emwas, A. H. M. (2015). The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. In *Metabonomics* (pp. 161–193). New York: Springer.

Farag, M. A., Porzel, A., & Wessjohann, L. A. (2012). Comparative metabolite profiling and fingerprinting of medicinal licorice roots using a multiplex approach of GC–MS, LC–MS and 1D NMR techniques. *Phytochemistry, 76*, 60–72.

Fiehn, O. (2002). Metabolomics—the link between genotypes and phenotypes. In *Functional genomics* (pp. 155–171). Heidelberg: Springer.

Gavaghan, C. L., Li, J. V., Hadfield, S. T., Hole, S., Nicholson, J. K., Wilson, I. D., et al. (2011). Application of NMR-based metabolomics to the investigation of salt stress in maize (*Zea mays*). *Phytochemical Analysis, 22*(3), 214–224.

Hall, R. D. (2006). Plant metabolomics: From holistic hope, to hype, to hot topic. *New Phytologist, 169*(3), 453–468.

Jäger, A. K., Light, M. E., & Van Staden, J. (1996). Effects of source of plant material and temperature on the production of smoke extracts that promote germination of light-sensitive lettuce seeds. *Environmental and Experimental Botany, 36*(4), 421–429.

Keeley, J. E. (1993). Smoke-induced flowering in the fire-lily *Cyrtanthus ventricosus. South African Journal of Botany, 59*(6), 638.

Keeley, J. E., Bond, W. J., Bradstock, R. A., Pausas, J. G., & Rundel, P. W. (2012). *Fire in Mediterranean ecosystems: Ecology, evolution and management*. Cambridge: Cambridge University Press.

Keeley, J. E., Pausas, J. G., Rundel, P. W., Bond, W. J., & Bradstock, R. A. (2011). Fire as an evolutionary pressure shaping plant traits. *Trends in Plant Science, 16*(8), 406–411.

Kim, E. J., Kwon, J., Park, S. H., Park, C., Seo, Y.-B., Shin, H.-K., et al. (2011). Metabolite profiling of *Angelica gigas* from different geographical origins using ^{1}H NMR and UPLC-MS analyses. *Journal of Agricultural and Food Chemistry, 59*(16), 8806–8815.

Koch, K. (2004). Sucrose metabolism: Regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology, 7*(3), 235–246.

Kohli, P., Kalia, M., & Gupta, R. (2015). Pectin methylesterases: A review. *Journal of Bioprocessing & Biotechniques, 5*(5), 1.

Komarova, T. V., Sheshukova, E. V., & Dorokhov, Y. L. (2014). Cell wall methanol as a signal in plant immunity. *Frontiers in Plant Science, 5*, 101.

Krishnan, P., Kruger, N. J., & Ratcliffe, R. G. (2005). Metabolite fingerprinting and profiling in plants using NMR. *Journal of Experimental Botany, 56*(410), 255–265.

Kulkarni, M. G., Sparg, S. G., Light, M. E., & Van Staden, J. (2006). Stimulation of rice (*Oryza sativa* L.) seedling vigour by smoke-water and butenolide. *Journal of Agronomy and Crop Science, 192*(5), 395–398.

LeClere, S., Schmelz, E. A., & Chourey, P. S. (2010). Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. *Plant Physiology, 153*(1), 306–318.

Light, M. E., Burger, B. V., & Van Staden, J. (2005). Formation of a seed germination promoter from carbohydrates and amino acids. *Journal of Agricultural and Food Chemistry, 53*(15), 5936–5942.

Light, M. E., Kulkarni, M. G., Ascough, G. D., & Van Staden, J. (2007). Improved flowering of a South African *Watsonia* with smoke treatments. *South African Journal of Botany, 73*(2), 298.

Lin, C. Y., Viant, M. R., & Tjeerdema, R. S. (2006). Metabolomics: Methodologies and applications in the environmental sciences. *Journal of Pesticide Science, 31*(3), 245–251.

López-Ibáñez, J., Pazos, F., & Chagoyen, M. (2016). MBROLE 2.0—functional enrichment of chemical compounds. *Nucleic Acids Research, 44*(W1), W201–W204.

Malabadi, R. B., & Nataraja, K. (2007). Smoke-saturated water influences somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana* AB Jacks. *Journal of Plant Sciences, 2*(1), 45–53.

Micheli, F. (2001). Pectin methylesterases: Cell wall enzymes with important roles in plant physiology. *Trends in Plant Science, 6*(9), 414–419.

Moreira, B., Tormo, J., Estrelles, E., & Pausas, J. G. (2010). Disentangling the role of heat and smoke as germination cues in Mediterranean Basin flora. *Annals of Botany, 105*(4), 627–635.

Nelson, D. C., Flematti, G. R., Ghisalberti, E. L., Dixon, K. W., & Smith, S. M. (2012). Regulation of seed germination and seedling growth by chemical signals from burning vegetation. *Annual Review of Plant Biology, 63*, 107–130.

Nelson, D. C., Flematti, G. R., Riseborough, J. A., Ghisalberti, E. L., Dixon, K. W., & Smith, S. M. (2010). Karrikins enhance light responses during germination and seedling development in *Arabidopsis thaliana. Proceedings of the National Academy of Sciences, 107*(15), 7095–7100.

Odunsi, K., Wollman, R. M., Ambrosone, C. B., Hutson, A., McCann, S. E., Tammela, J., et al. (2005). Detection of epithelial ovarian cancer using ^{1}H-NMR-based metabonomics. *International Journal of Cancer, 113*(5), 782–788.

Pan, Z., & Raftery, D. (2007). Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Analytical and Bioanalytical Chemistry, 387*(2), 525–527.

Papenfus, H. B., Kumari, A., Kulkarni, M. G., Finnie, J. F., & Van Staden, J. (2014). Smoke-water enhances in vitro pollen germination and tube elongation of three species of Amaryllidaceae. *South African Journal of Botany, 90*, 87–92.

Pelloux, J., Rusterucci, C., & Mellerowicz, E. J. (2007). New insights into pectin methylesterase structure and function. *Trends in Plant Science, 12*(6), 267–277.

Pierce, S. M., Esler, K., & Cowling, R. M. (1995). Smoke-induced germination of succulents (Mesembryanthemaceae) from fire-prone and fire-free habitats in South Africa. *Oecologia, 102*(4), 520–522.

Rehman, A., ur Rehman, S., Khatoon, A., Qasim, M., Itoh, T., Iwasaki, Y., et al. (2018). Proteomic analysis of the promotive effect of plant-derived smoke on plant growth of chickpea. *Journal of Proteomics, 176*, 56–70.

Rolland, F., Baena-Gonzalez, E., & Sheen, J. (2006). Sugar sensing and signaling in plants: Conserved and novel mechanisms. *Annual Review of Plant Biology, 57*, 675–709.

Rosa, M., Prado, C., Podazza, G., Interdonato, R., González, J. A., Hilal, M., et al. (2009). Soluble sugars: Metabolism, sensing and abiotic stress: A complex network in the life of plants. *Plant Signaling & Behavior, 4*(5), 388–393.

Ruan, Y. L. (2012). Signaling role of sucrose metabolism in development. *Molecular Plant, 5*(4), 763–765.

Savorani, F., Rasmussen, M. A., Mikkelsen, M. S., & Engelsen, S. B. (2013). A primer to nutritional metabolomics by NMR spectroscopy and chemometrics. *Food Research International, 54*(1), 1131–1145.

Senaratna, T., Dixon, K., Bunn, E., & Touchell, D. (1999). Smoke-saturated water promotes somatic embryogenesis in geranium. *Plant Growth Regulation, 28*(2), 95–99.

Singh, S., Kulkarni, M. G., & Van Staden, J. (2014). Biochemical changes associated with gibberellic acid-like activity of smoke-water, karrikinolide and vermicompost leachate during seedling development of *Phaseolus vulgaris* L. *Seed Science Research, 24*(1), 63–70.

Singh, V., van Oosterom, E. J., Jordan, D. R., Messina, C. D., Cooper, M., & Hammer, G. L. (2010). Morphological and architectural development of root systems in sorghum and maize. *Plant and Soil, 333*(1–2), 287–299.

Smeekens, S., & Rook, F. (1997). Sugar sensing and sugar-mediated signal transduction in plants. *Plant Physiology, 115*(1), 7.

Song, J., Liu, C., Li, D., & Gu, Z. (2013). Evaluation of sugar, free amino acid, and organic acid compositions of different varieties of vegetable soybean (*Glycine max* [L.] Merr). *Industrial Crops and Products, 50*, 743–749.

Soós, V., Juhász, A., Light, M. E., Van Staden, J., & Balázs, E. (2009b). Smoke-water-induced changes of expression pattern in Grand Rapids lettuce achenes. *Seed Science Research, 19*(1), 37–49.

Soós, V., Sebestyén, E., Juhász, A., Light, M. E., Kohout, L., Szalai, G., et al. (2010). Transcriptome analysis of germinating maize kernels exposed to smoke-water and the active compound KAR_1. *BMC Plant Biology, 10*(1), 236.

Soós, V., Sebestyén, E., Juhász, A., Pintér, J., Light, M. E., Van Staden, J., et al. (2009a). Stress-related genes define essential steps in the response of maize seedlings to smoke-water. *Functional & Integrative Genomics, 9*(2), 231–242.

Soós, V., Sebestyén, E., Posta, M., Kohout, L., Light, M. E., Staden, J., et al. (2012). Molecular aspects of the antagonistic interaction of smoke-derived butenolides on the germination process of Grand Rapids lettuce (*Lactuca sativa*) achenes. *New Phytologist, 196*(4), 1060–1073.

Sparg, S. G., Kulkarni, M. G., & Van Staden, J. (2006). Aerosol smoke and smoke-water stimulation of seedling vigor of a commercial maize cultivar. *Crop Science, 46*(3), 1336–1340.

Staden, J. V., Brown, N. A., Jäger, A. K., & Johnson, T. A. (2000). Smoke as a germination cue. *Plant Species Biology, 15*(2), 167–178.

Sturm, A., & Tang, G.-Q. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science, 4*(10), 401–407.

Van Staden, J., Sparg, S. G., Kulkarni, M. G., & Light, M. E. (2006). Post-germination effects of the smoke-derived compound 3-methyl-2*H*-furo[2, 3-*c*]pyran-2-one, and its potential as a preconditioning agent. *Field Crops Research, 98*(2–3), 98–105.

Wang, M., Schoettner, M., Xu, S., Paetz, C., Wilde, J., Baldwin, I. T., et al. (2017). Catechol, a major component of smoke, influences primary root growth and root hair elongation through reactive oxygen species-mediated redox signaling. *New Phytologist, 213*(4), 1755–1770.

Weljie, A. M., Newton, J., Mercier, P., Carlson, E., & Slupsky, C. M. (2006). Targeted profiling: Quantitative analysis of ^{1}H NMR metabolomics data. *Analytical Chemistry, 78*(13), 4430–4442.

Wishart, D. S. (2008). Quantitative metabolomics using NMR. *TrAC Trends in Analytical Chemistry, 27*(3), 228–237.

Wolf, S., Mouille, G., & Pelloux, J. (2009). Homogalacturonan methyl-esterification and plant development. *Molecular Plant, 2*(5), 851–860.

Wu, H., Southam, A. D., Hines, A., & Viant, M. R. (2008). High-throughput tissue extraction protocol for NMR-and MS-based metabolomics. *Analytical Biochemistry, 372*(2), 204–212.

Xia, J., & Wishart, D. S. (2016). Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Current Protocols in Bioinformatics*, 55, 14.10.1–14.10.91.

Yearsley, E. M., Fowler, W. M., & He, T. (2018). Does smoke water enhance seedling fitness of serotinous species in fire-prone south-western Western Australia? *South African Journal of Botany, 115*, 237–243.

Zhang, A., Sun, H., Qiu, S., & Wang, X. (2013). NMR-based metabolomics coupled with pattern recognition methods in biomarker discovery and disease diagnosis. *Magnetic Resonance in Chemistry, 51*(9), 549–556.

Zhang, A., Sun, H., Wang, P., Han, Y., & Wang, X. (2012). Modern analytical techniques in metabolomics analysis. *Analyst, 137*(2), 293–300.

Zhi, H.-J., Qin, X.-M., Sun, H.-F., Zhang, L.-Z., Guo, X.-Q., & Li, Z.-Y. (2012). Metabolic fingerprinting of *Tussilago farfara* L. using ^{1}H-NMR spectroscopy and multivariate data analysis. *Phytochemical Analysis, 23*(5), 492–501.

Zhu, J., Mickelson, S. M., Kaeppler, S. M., & Lynch, J. P. (2006). Detection of quantitative trait loci for seminal root traits in maize (*Zea mays* L.) seedlings grown under differential phosphorus levels. *Theoretical and Applied Genetics, 113*(1), 1–10.