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SHORT COMMUNICATION

Jasmonic acid to boost secondary growth in hemp hypocotyl

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Abstract

Main conclusion **The application of jasmonic acid results in an increased secondary growth, as well as additional secondary phloem fibres and higher lignin content in the hypocotyl of textile hemp (*Cannabis sativa* L.).**

Secondary growth provides most of the wood in lignocellulosic biomass. Textile hemp (*Cannabis sativa* L.) is cultivated for its phloem fibres, whose secondary cell wall is rich in crystalline cellulose with a limited amount of lignin. Mature hemp stems and older hypocotyls are characterised by large blocks of secondary phloem fibres which originate from the cambium. This study aims at investigating the role of exogenously applied jasmonic acid on the differentiation of secondary phloem fibres. We show indeed that the exogenous application of this plant growth regulator on young hemp plantlets promotes secondary growth, differentiation of secondary phloem fibres, expression of lignin-related genes, and lignification of the hypocotyl. This work paves the way to future investigations focusing on the molecular network underlying phloem fibre development.

Keywords Bast fibre · Cambium · Cell wall · Gene expression · Lignin

Abbreviation
JA Jasmonic acid

Introduction

Shoot primary growth results from the activity of the shoot apical meristem and leads to the development of primary tissues in the stem, leaf, and hypocotyl. At the end of the elongation phase of the stem and hypocotyl, further development occurs by secondary growth. This process results from the activity of the vascular cambium. The cambium initials divide periclinally to produce xylem (inwards) and phloem (outwards) mother cells, and anticlinally to create new initials to follow the increased circumference of the stem (Nieminen et al. 2015). This process is particularly obvious in perennial species such as trees. As described in aspen using high-spatial-resolution RNA-Seq analysis, complex gene networks and modules are associated with vascular cambium, secondary phloem, and secondary xylem development (Sundell et al. 2017). Secondary growth is also occurring in some annual herbaceous plants, such as *Arabidopsis thaliana*, where it has been studied both in the inflorescence stem (Ko et al. 2004) and in the hypocotyl (Ragni and Hardtke 2014). In the hypocotyl system of both *Arabidopsis thaliana* and other herbaceous plants like fibre crops, secondary growth occurs quickly, roughly 10 days after sowing, and elongation and secondary thickening are temporally uncoupled (Chaffey et al. 2002; Baucher et al. 2007; Roach and Deyholos 2008; Behr et al. 2016). Procambium proliferation and cambium identity are tuned by the dodecapeptide TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) which is secreted from the phloem (Nieminen et al. 2015). TDIF induces the expression of the transcription factor WUSCHEL-RELATED HOMEOBOX 4 (WOX4).

Phytohormones are key molecules regulating secondary growth (Strabala and MacMillan 2013; Ursache et al. 2013).

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The role of auxin has long been proven (Aloni 2013). The development of the vascular cambium is severely affected when the shoot tip is decapitated, causing the loss of the main auxin source in the plant. In addition, secondary growth triggered by the perception of a weight signal induces the expression of many genes having auxin responsive elements in their promoter region, possibly indicating a role for auxin in this process (Ko et al. 2004). Auxin has also been shown to promote cambial cell division by TDIF-WOX4 and other signalling pathways (Suer et al. 2011; Nieminen et al. 2015; Brackmann et al. 2018). Mutants impaired in cytokinin biosynthesis have strong phenotypes, with stem dwarfism and greatly reduced diameter (Nieminen et al. 2015). Cytokinin receptors are required for the correct development of vascular tissues in the hypocotyl (Kuroha et al. 2006). Floral induction and/or growth of the inflorescence stem trigger secondary growth in the hypocotyl by basipetally translocated gibberellins (Sibout et al. 2008; Strabala and MacMillan 2013). Gibberellins also stimulate cambial activity, induce fibre differentiation (Aloni 2013), and trigger hypocotyl xylem expansion (Ragni et al. 2011). Finally, jasmonates positively regulate secondary growth through the canonical signalling SCF-CORONATINE INSENSITIVE 1 (SCF^{COI1}) pathway. Silencing the negative regulator of jasmonate signalling *JASMONATE-ZIM-DOMAIN PROTEIN 10* (*JAZ10*) results in an earlier and more pronounced interfascicular cambium activity in *Arabidopsis* and exogenous application of jasmonic acid on stem triggers the formation of phloem fibres (a.k.a. bast fibres) in wild-type plants (Sehr et al. 2010).

Lignification is the last step of vascular cell differentiation. Lignin impregnates the extracellular matrix (compound middle lamella and secondary cell wall) of phloem fibres, xylem fibres, and xylem vessels (Barros et al. 2015). The timing and the subcellular localisation of lignin deposition are tightly controlled by specific transcription factors (Wang and Dixon 2012; Zhong and Ye 2012; Sakamoto and Mitsuda 2015) and interactions between phytohormones (Aloni et al. 1990; Hentrich et al. 2013; Didi et al. 2015; Nieminen et al. 2015) which are specific for the plant developmental stage. Since lignin provides mechanical strength and resistance to gravity to the stem, lignification is extremely important for the ability of the plant to cope with these factors. Phytohormones, e.g., auxin and jasmonic acid, affect gene expression patterns (Ko et al. 2004; Sehr et al. 2010) and up-regulate specific genes (*PAL*, *4CL*, *CCR*, *CCoAOMT,* and *CAD*) of the monolignol pathway (Pauwels et al. 2008; Tamaoki et al. 2011), thereby affecting plant development.

In the hemp stem, fibres are either associated with xylem or with phloem (Schuetz et al. 2013), of both primary (procambium) and secondary (cambium) origins. Xylem fibres (known as hurds or shivs) possess a xylan-type secondary cell wall, and they are rich in lignin (Gorshkova et al. 2012) and reported to contain silica. Silica binds well with lime, therefore favouring the compaction of the hurds and the formation of a concrete-like material to be used in the construction industry (e.g., production of a lightweight material known as "hempcrete", Luyckx et al. 2017). Phloem fibres are characterised by their extreme length (up to 55 mm) and possess gelatinous-type cell walls (Snegireva et al. 2015) with a noteworthy thickness and high-crystalline cellulose content (up to 75–80%; Guerriero et al. 2013). They are used for the manufacturing of commercial textiles and composite materials (Gorshkova et al. 2012). Secondary phloem fibres produced by cambial activity in fully developed internodes are far more abundant than primary phloem fibres (Blake et al. 2008). Secondary phloem fibres are shorter and more lignified than the primary ones (Snegireva et al. 2015; Fernandez-Tendero et al. 2017), causing heterogeneity in the industrial quality of hemp technical fibres (Liu et al. 2015).

We previously showed some of the factors involved in hemp secondary growth using the hypocotyl system (Behr et al. 2016). The bioactive jasmonate content and the expression of some genes involved in secondary growth (*WOX4*, *TDIF RECEPTOR*) increased at the onset of cambial activity and secondary tissue formation, leading to the up-regulation of the secondary cell wall-related genes and further thickening of the fibres (cellulose, lignin, and xylan deposition). This is particularly interesting, especially if one considers the recent hypothesis, we put forward concerning the role of JA in hemp bast fibre growth (Guerriero et al. 2017): more specifically, this phytohormone may regulate intrusive growth together with indole glucosinolates and, therefore, be determinant in early phases of bast fibre development.

The present study aims at investigating more thoroughly the function of jasmonates on bast fibre differentiation and lignification. *Arabidopsis* produces secondary phloem fibres in reduced quantities (Altamura et al. 2001) as compared to hemp. We thus choose this fibre crop to further study the role of jasmonates in secondary phloem fibre differentiation and overall hypocotyl lignification. Hypocotyls aged 15 days (corresponding to the maximum endogenous jasmonate content, Behr et al. 2016) were sprayed either with JA or with a mock solution. We reasoned that increasing the jasmonate content (via exogenous application) in the plant at the stage where the endogenous level reached its maximum, may amplify the response of the plant towards this phytohormone, highlighting its functions in this biological context. The resulting differences in terms of secondary growth and lignification patterns are presented to shed light on the biological mechanism leading to lignification and secondary phloem fibres biogenesis in this important fibre crop.

Materials and methods

Experimental setup

Hemp (*Cannabis sativa* L.) hypocotyls of the fibre cv Santhica 27 were grown in a mixture of compost/sand (1:1, w/w) in controlled conditions (Behr et al. 2016). Seeds were provided by the Coopérative Centrale des Producteurs de Semences de Chanvre (Beaufort-en-Vallée, France) and certified by the Service Officiel de Contrôle et de Certification (SOC) of the Groupement National Interprofessionnel des Semences et plants (GNIS), France. Either JA, at a concentration ranging from 0.03 to 3 mM, or the mock solution was sprayed on plantlets aged 15 days after sowing (1 mL plant^{-1}). The JA solution (Sigma-Aldrich, J2500) was prepared in phosphate buffer saline (PBS) from a 500-fold stock in pure ethanol. A mock solution, consisting of PBS with a minute amount of ethanol (0.06% in the sprayed solution) compensating for its presence in the jasmonic acid solution, was sprayed on control plantlets.

Lignin analysis

For lignin analysis, 5 biological replicates, each consisting of a pool of 12 hypocotyls, were sampled 3 days after the treatment and used to prepare the cell wall residue (CWR). Hypocotyls were reduced to a fine powder using liquid nitrogen, a mortar, and a pestle. CWR was obtained by washing the powdered material first with methanol (80%) under agitation for 4 h, followed by five additional washing with ethanol (80%) to obtain a residue free of soluble molecules. Lignin content was determined with the acetyl bromide method using 5 mg of dried CWR (Hatfield et al. 1999). The CWR was digested with 2.6 mL of 25% acetyl bromide in glacial acetic acid for 2 h at 50 °C using a Hach LT200 system. At the end of the reaction, the solution was transferred to a 50 mL Falcon tube containing 10 mL of 2 M sodium hydroxide and 12 mL of glacial acetic acid. After rinsing the reaction tube with glacial acetic acid, 1.75 mL of 0.5 M hydroxylammonium chloride was added and the total volume adjusted to 30 mL with glacial acetic acid. The absorbance at 280 nm was read with a spectrophotometer. An extinction coefficient of 22.9 g^{-1} L cm^{-1} was used to calculate the lignin content.

Microscopic observations

For the microscopic analysis, a second batch of hypocotyls treated with JA at 0.1 mM was harvested 5 days after the treatment (corresponding to hypocotyls aged 20 days). We anticipated an enhanced secondary growth, and resulting secondary tissues formation, caused by the application of JA to be more important after 5 days, rather than 3 days after the treatment (corresponding to hypocotyls aged 18 days). Indeed, we have observed, in hemp hypocotyls, that the formation of secondary phloem fibres occurs massively between 15 and 20 days (Behr et al. 2018). The average diameter of 32–33 hypocotyls was determined with a digital caliper for the two treatments (JA at 0.1 mM and mock spraying), and a subset of 12 hypocotyls representative of the diameter of each batch was observed 5 days after application (i.e., when the plantlets were 20-day-old, Online Resource S1). Hypocotyls were harvested, embedded in 5% (w/v) agarose, cut at a thickness of 100 µm using a VT1000 S vibratome (Leica Biosystems), and observed with a confocal microscope system (LSM 880, Zeiss) with the following settings: excitation at 488 and 543 nm and emission recorded in the BP 505–570 nm and LP 560 nm. The secondary phloem fibres were counted manually in a quarter of the hypocotyl cross section (similar to Snegireva et al. 2015). Fixed, resin-embedded cross sections of hypocotyls (JA or mock treatment) were also stained with FASGA and observed with a light microscope, as described in (Behr et al. 2018).

RNA extraction and RT-qPCR

Gene expression analysis was performed on hypocotyls aged 16 (H16), 17 (H17), 18 (H18), and 20 (H20) days after sowing by RT-qPCR. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and quality-checked using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and a 2100 Bioanalyzer (Agilent Life Sciences). Reverse transcription was carried out with the ProtoScript II Reverse Transcriptase (NEB) following the manufacturer's instructions. Primers were previously validated (Behr et al. 2016, 2017). RT-qPCR runs were performed in 384 well plates, on a ViiA7 Real-Time PCR System (Applied Biosystems) with the Takyon SYBR Green low ROX (Eurogentec). A melt curve was realised at the end of each experiment to check the specificity of the products. Relative gene expressions were determined with the qBasePLUS software v2.5 (Biogazelle).

Results and discussion

This study aims at describing the influence of exogenous JA on secondary growth and lignification. To this end, this plant growth regulator was sprayed on hemp plantlets 15 days after sowing. The resulting phenotype was quantitatively characterised by measuring three variables in the hypocotyl: its lignin content, its diameter, and the number of secondary phloem fibres.

As shown in Fig. 1, JA has a positive effect on the lignin content of the hypocotyls, with the exception of the 1 mM

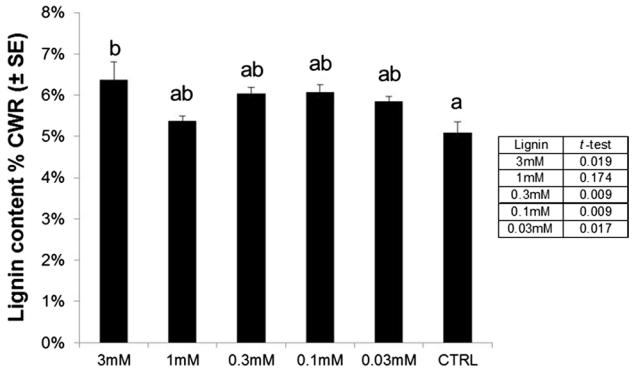

Fig. 1 Impact of JA application on hypocotyl lignin content (average ± standard error of the mean, SE, $n = 5$). The letters correspond to homogeneous groups as assessed by Tukey post-hoc test. A Student's *t*-test is performed for each concentration (Table). *CTRL* mock control, *CWR* cell wall residue

concentration for which the statistical test did not detect significant changes (*t*-test *p*-value = 0.174). For instance, the application of 0.1 mM JA resulted in a ca. 20% increase of the lignin content as compared to the mock (6.1 vs. 5.1%).

Next, the effect of JA on secondary growth was assessed through its impact on the diameter of the hypocotyl. As for lignin analysis, the hypocotyls were treated with either one of the five concentrations of JA or with a mock solution, and measured 3 days after. Under these conditions, JA enhanced secondary growth when it was applied at a concentration of 0.03, 0.1, and 0.3 mM (Fig. 2a). The statistically significant increase in the hypocotyl diameters was confirmed with two independent experiments using > 30 and a subset of 12 hypocotyls (Online Resource S1). At higher concentrations (1 and 3 mM), we were not able to detect any positive effect on this variable; as previously reported in *Arabidopsis* cell suspension culture, higher JA concentrations (200 µM instead of 50 µM) may significantly slow down cell cycle progression during the mitotic stage, resulting in a high proportion of cells in the G_2 phase (Pauwels et al. 2008), leading to attenuated cambial activity. Therefore, this may explain the negative effect of the 3 mM modality on the hypocotyl diameter. However, our data overall confirm the previously reported positive role of JA on secondary growth. Indeed, Sehr et al. (2010) showed that JA-treated *Arabidopsis* stem displayed a higher lateral interfascicular cambium-derived tissue (ICD) extension, while mutants deficient in JASMONATE-ZIM DOMAIN (JAZ) protein showed higher lateral ICD and stem diameter. The proteins from the JAZ family are negative regulators of JA signalling (De Geyter et al. 2012). Recently, several genes involved in the biosynthesis and signalling of JA were found to be highly expressed during secondary growth, in both the wild type and the *suppressor of overexpression of constans 1* (*soc1*) and *fruitfull* (*ful*) woody (*soc1ful*) mutant of thale cress (Melzer et al. 2008; Davin et al. 2016). The *soc1ful* mutant displays a woody phenotype, consisting in a closed cambial ring and a large production of wood. These results, therefore, suggest that, in hemp, JA signalling mediates secondary growth through the up-regulation of genes involved in cambial activity. In addition, we have observed that three genes involved in JA biosynthesis (two being annotated as *lipoxygenase 2* and one as *4-coumarate-CoA ligase-like 7*) were 4- to 31-times more expressed in hemp basal phloem fibres (vs. elongating phloem fibre), where secondary tissues from the cambium are well visible (Guerriero et al. 2017). The impact of JA treatment on hemp hypocotyl diameters is less obvious than the phenotypes reported in the previous works (Melzer et al. 2008; Sehr et al. 2010), presumably because this experimental setup, consisting in a single application of JA on young plantlets, is less powerful than the mutants used in these studies. Our conclusion that

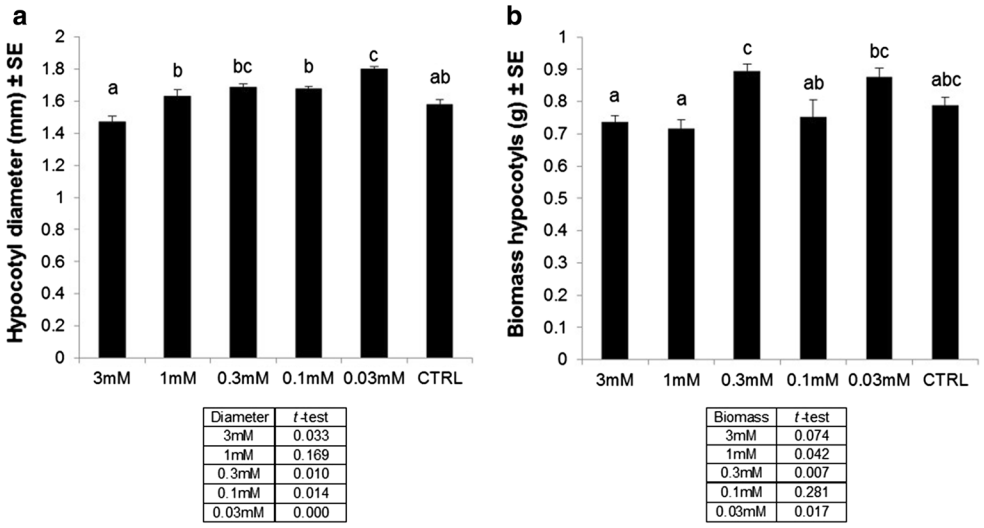

Fig. 2 Impact of JA application on hypocotyl diameter (**a**) and biomass production (mass of 12 hypocotyls, **b**) (average ± SE). The letters correspond to homogeneous groups as assessed by Tukey post-hoc test. A Student's *t*-test is performed for each concentration (Tables)

JA favours secondary growth in hemp may, therefore, be strengthened by manipulating JA biosynthesis or signalling. The biomass was not significantly increased or decreased by JA application (Fig. 2b), possibly due to the variation in hypocotyl length.

Jasmonates are known to induce the secondary metabolism through the up-regulation of specific transcription factors such as *WRKY*s, *MYB*s, and *MYC*s (De Geyter et al. 2012; Zhou and Memelink 2016). More precisely, JA positively regulates monolignol biosynthesis, as shown in *Arabidopsis* cells grown in suspension (Pauwels et al. 2008). RT-qPCR on control and hypocotyls treated with 0.1 mM (a concentration showing both increased lignin content, Fig. 1 and diameter, Fig. 2a) analysis suggests that some genes of the phenylpropanoids/monolignols pathway are indeed up-regulated by JA (Fig. 3). The hypocotyls may, therefore, biosynthesise more monolignols, being polymerised into the lignin polymer in the apoplast. In addition to monolignol biosynthesis, jasmonates directly or indirectly regulate the expression of several cell wall-related genes (Sánchez-Rodríguez et al. 2010). JA treatment may, therefore, modify the polysaccharide composition of the cell wall.

Finally, our last experiment aimed at studying whether the increased hypocotyl diameter results in a higher number of secondary phloem fibres. In their study, Sehr et al. (2010) observed that wild-type *Arabidopsis* stems treated with JA have additional phloem fibres. To quantitatively characterise this phenomenon, we have counted, in an independent experiment, the secondary phloem fibres in mock- and JA-treated (0.1 mM) hypocotyls (12 plantlets for each modality). Cross sections of JA-treated plantlets displayed significantly more secondary phloem fibres than the mock-treated (505 vs. 341, *t*-test *p*-value 0.93%, see Fig. 4 and Online Resource S1). The area covered by the secondary phloem fibres was higher in JA-treated hypocotyls (*t*-test *p*-value 2.68%), while the secondary xylem was more extended in mock-treated hypocotyls (*t*-test *p*-value 2.93%, Fig. 4 and Online Resource S1). This result strongly suggests that secondary xylem and phloem development is regulated by different phytohormones in hemp. For instance, gibberellins are involved in the xylogenesis of *Arabidopsis* hypocotyl (Ragni et al. 2011). The expression level of several genes regulating xylem development (*VND6*, *VND7*, *VNI2*, *MYB46*, *MYB58*, *MYB85*, *PXC1,* and *XND1*) was not significantly altered by JA (data not shown), suggesting that the functions of JA are not directly linked with xylem development. Representative pictures of the hypocotyls relative to the two treatments are depicted in Fig. 4.

The increased differentiation of secondary phloem fibres in JA-treated hypocotyls is consistent with the link between JA, secondary growth, and mechanical cues (increased strength needed to mechanically support the stem). Secondary growth has been positively associated with the loss of the *soc1* and *ful* genes, causing higher intra-tissue tension related to broad wood formation (Davin et al. 2016). This double mutant is characterised by the loss of its herbaceous habitus, up-regulation of the genes of the JA pathways and enhanced xylogenesis. Likewise, mechanical "stimulation", such as that associated with the increased stem weight resulting from secondary growth, is also associated with the up-regulation of touch-inducible and stress-related genes (Sehr et al. 2010). The perception of these signals leads to the induction of the JA biosynthetic and signalling pathways (Ragni and Greb 2017). Ultimately, the plantlet facing these cues develops a more vigorous phenotype, through an enhanced secondary growth.

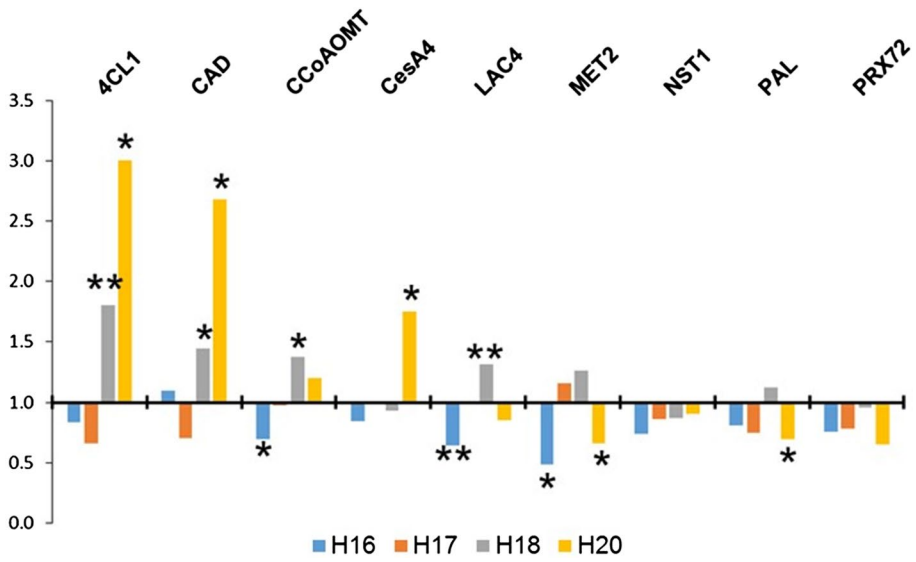

Fig. 3 Gene expression between H16 and H20. The values are fold-change between JA-treated (0.1 mM) and mock-treated hypocotyls. Values above 1 indicate genes more expressed in JA-treated hypocotyls (*t*-test *p*-value < 0.05* and < 0.01**). *4CL1* 4-coumaric acid-CoA ligase 1, *CAD* (hydroxy)cinnamyl alcohol dehydrogenase, *CCoAOMT* caffeoyl-CoA 3-*O*-methyltransferase, *CesA4* cellulose synthase 4, *LAC4* laccase 4, *MET2* methionine synthase 2, *NST1* NAC secondary wall thickening promoting factor 1, *PAL* phenylalanine ammonia lyase, *PRX72* peroxidase 72

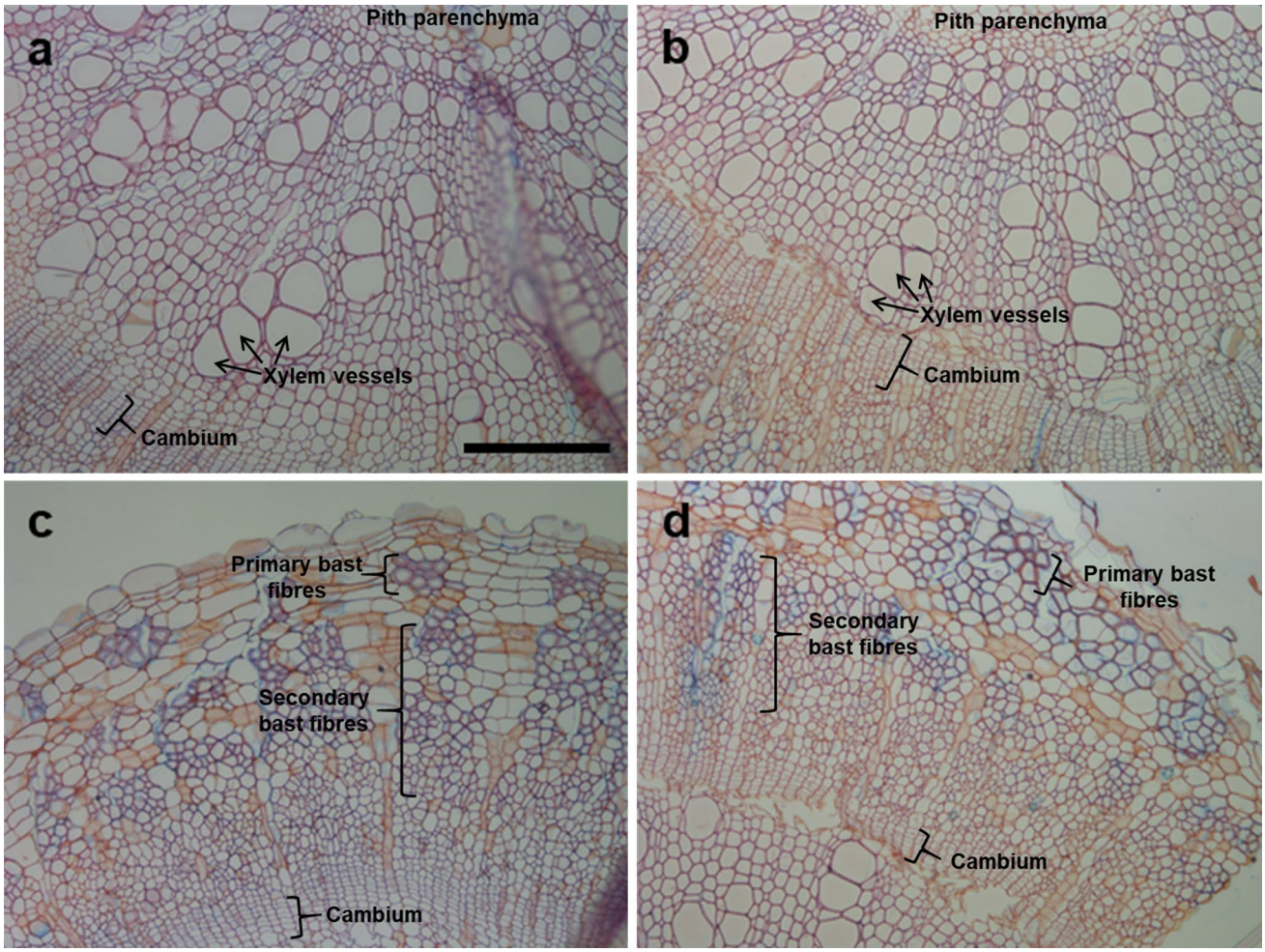

Fig. 4 Representative cross section of mock- and JA-treated 20-day-old hypocotyls stained with FASGA. **a**, **c** Details of the xylem and phloem of a mock-treated hypocotyl. **b**, **d** Details of the xylem and phloem of a JA-treated hypocotyl. Scale bar (in black and relative to all the panels) = 200 µm. The different cell types are indicated

Conclusion

Secondary growth is important in relation with the production of secondary fibres in hemp. We have here provided evidence that JA stimulates this process and leads to the formation of additional secondary phloem fibres in the hypocotyl of textile hemp. In addition, the lignin content was significantly enhanced by JA.

Author contribution statement MB, SL, and JFH designed the study. MB performed all experiments and wrote the manuscript. SL, JFH, and GG critically revised the manuscript with interpretation inputs. All authors read and approved the manuscript.

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Compliance with ethical standards

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

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