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Enhanced production of lignans and neolignans in chitosan-treated flax (*Linum usitatissimum* L.) cell cultures

Waqar Ahmad[a], Adnan Zahir[a], Muhammad Nadeem[a], Laurine Garros[b,d,e], Samantha Drouet[b,d], Sullivan Renouard[b,d], Joël Doussot[b,d,f], Nathalie Giglioli-Guivarc'h[c,d], Christophe Hano[b,d,*], Bilal Haider Abbasi[a,b,c,d,*]

[a] Department of Biotechnology, Quaid-i-Azam University, Islamabad 45320, Pakistan
[b] Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC), Plant Lignans Team, INRA USC1328, Université d'Orléans, F 28000 Chartres, France
[c] EA2106 Biomolécules et Biotechnologies Végétales, Université François-Rabelais de Tours, Tours, France
[d] Bioactifs et Cosmétiques, GDR 3711 COSMACTIFS, CNRS/Université d'Orléans, France
[e] Institut de Chimie Organique et Analytique, ICOA UMR7311, Université d'Orléans-CNRS, 45067 Orléans CÉDEX 2, France
[f] Le CNAM, Conservatoire National des Arts et Métiers, CASER-SITI-CG, 2 rue Conté, 75003 Paris, France

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ABSTRACT

Linum usitatissimum is a source of pharmacologically active lignans and neolignans. An effective protocol has been established for the enhanced biosynthesis of lignans and neolignans in cell cultures of *Linum usitatissimum* by using chitosan addition. Gene expression analysis of monolignols (*PAL*, *CCR* and *CAD*), lignans (*DIR*, *PLR* and *UGT*) and neolignans (*PCBER*) biosynthetic genes by RT-qPCR as well as monolignol biosynthetic PAL, CCR and CAD enzyme activities evidenced a stimulation following chitosan treatment. Validated reverse phase high-performance liquid chromatography coupled to diode array detection was used to quantify secoisolariciresinol diglucoside (SDG) and lariciresinol diglucoside (LDG), dehydrodiconiferyl alcohol glucoside (DCG) and guaiacylglycerol-β-coniferyl alcohol ether glucoside (GGCG) showed that chitosan treated cell cultures had better accumulation of these metabolites. Maximum enhancements of 7.3-fold (28 mg/g DW) occurred for LDG, 3.5-fold (58.85 mg/g DW) in DCG and while the least enhancement of 2-fold (18.42 mg/g DW) for SDG was observed in 10 mg/l chitosan treated cell cultures than to controls. Furthermore, same concentration of chitosan also resulted in 1.3-fold increase in antioxidant activity. Compared to the lignans and neolignans accumulations observed in wild type and RNAi-PLR transgenic flaxseeds, chitosan-treated cell cultures appeared to be a very effective production system for these compounds.

1. Introduction

Flax (*Linum usitatissimum* L.) is used for more than 10,000 years in terms of consumption and cultivation [1,2]. More recently, the detection of polyphenols (lignans, neolignans) has opened new dimensions for flax in pharmaceutical and nutraceutical industries to be used for preventing cancers, cardiovascular disorders, diabetes, lupus nephritis and other disorders [3]. Along with lignans, *L. usitatissimum* is considered a good source for accumulation of neolignans which are potential candidates in anti-inflammatory and antifungal applications [4].

Haworth coined the term lignan for the first time to define a class of dimeric phenylpropanoids in which two units C_6-C_3 are linked by the central C_8 [5], lignans are a group of phytochemicals (polyphenols) which are formed by connection of 2 cinnamyl alcohols [6]. Whereas Gottlieb speculated that molecules having 2 phenylpropanoid parts attached in a different fashion, such as C5-C5′ should be called neolignans [7]. Lignans and neolignans are structurally made of two propyl benzene units (Fig. 1). In lignans these units are associated by a β-β′ bond while this substitution pattern is different in neolignans [8]. Lignans are reported in more than 65 families of vascular plants, and are extracted from almost all parts of a plant [9].

Pharmacological activities of lignans have been demonstrated in various experimental models. Lignans induce the synthesis of 2-hydroxy estrogen in females which has a potential role in deterrence of cancer [10]. Lignans are believed to be responsible for inhibiting the growth of different human prostate cancer cell lines [11,12] According to reported data [13] when a dose of 10 mg/kg of lignans administered subcutaneously in athymic mice, have diminished the proliferation of

* Corresponding author.
E-mail addresses: christophe.hano@univ-orleans.fr (C. Hano), bhabbasi@qau.edu.pk (B.H. Abbasi).

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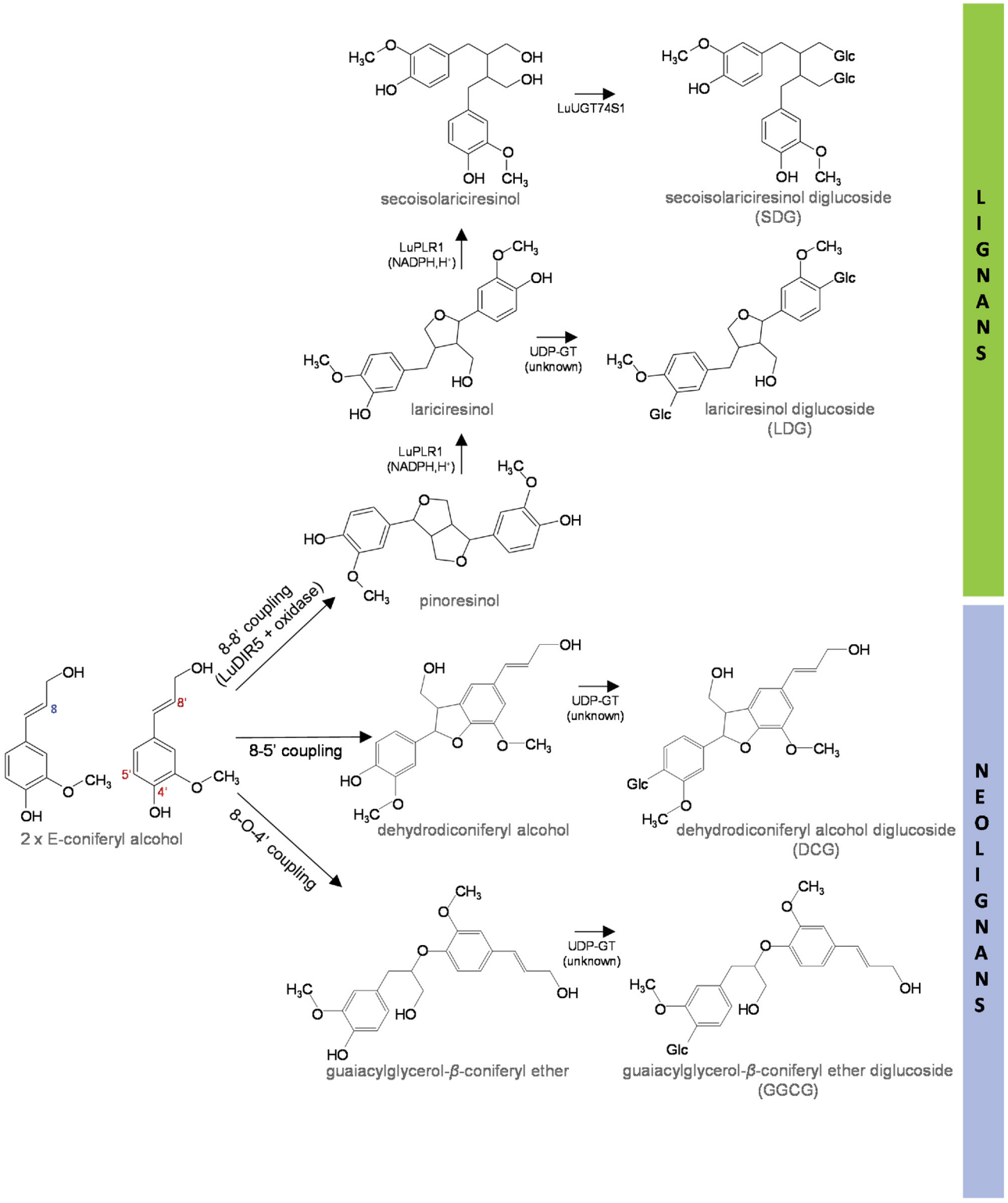

Fig. 1. Schematic representation of biosynthetic pathways of lignans and neolignans, quantified in cell cultures of flax using RP-HPLC-DAD adopted from Anjum et al.,[4] DIR: dirigent protein; PLR: pinoresinol lariciresinol reductase; UGT/UDP-GT: UDP-Glucosyl Transferase.

201 human colon cancer cell lines. Flax was also reported to abate the elevated blood glucose concentration in post meal duration [14,15]. A study conducted on a group of females in which they were fed with ground flax seeds 50 g/day, after four weeks their blood glucose level has been significantly reduced [16].

The production of secondary metabolites in different plant culture techniques using biotechnological interventions is an attractive substitute to the isolation from whole plant materials [16]. Using plant cell cultures coupled with elicitation strategies such as introduction of physical and chemical entities to the cell culture *per se* or growth conditions, as a biological platform for the synthesis of secondary metabolites is a promising approach for the sustainable production in modern medicinal and aromatic industry [17].

Due to growing interests on lignans and neolignans, alternate

platforms for their production other than conventional plant cultivation are needed. Cell and tissue culture techniques could be used for the enhanced biosynthesis of these metabolites. Chitosan is a polycationic β-1,4 linked D-glucosamine polymer known to act as a bioactive antifungal agent [18] though the elicitation of pathogenesis-related proteins in the host [19] and stimulation of phytoalexin production [20]. Chitosan has not been exploited previously for the accumulation of these valuable lignans and neolignans in cell cultures of flax. Therefore, in current study effect of chitosan is evaluated based on its impact on the biosynthesis and dynamics of these anti-cancer lignans and neolignans. A validated reverse-phase high performance liquid chromatography (RP-HPLC DAD) was used for the quantification of lignans and neolignans. As per our knowledge, this is the first report on enhanced biosynthesis of lignans (secoisolariciresinol diglucoside (SDG) and lariciresinol diglucoside (LDG)) and neolignans (dehydrodiconiferyl alcohol diglucoside (DCG) and guaiacylglycerol-β-coniferyl ether diglucoside (GGCG)) using chitosan as a stimulator of lignans and neolignans production in flax cell cultures. Current research has a potential to be scaled up to bioreactor levels for the feasible biosynthesis of these commercially important metabolites.

2. Materials and methods

2.1. Plant materials

Flax seeds (brown variety) were collected from malakand division hills (natural habitat) Khyber Pakhtunkhwa, Pakistan. The flax cultivar Barbara was supplied by Coopérative Terre de Lin (St Pierre le Viger, France). The generation of the RNAi plants and the homozygous transgenic lines were obtained as previously described [21].

For germination, seeds were surface sterilized using 0.1% mercuric chloride and 70% ethanol for 30 and 60 s respectively, subsequently washed 3 times with dH2O (autoclaved water) and dried using sterilized filter paper sheet. The sterilized seeds were inoculated on MS (Murashige and Skoog basal Media) following the protocol described earlier [22]. Growth room temperature was maintained at 25 ± 2 °C and 16/8 h (light/dark) photoperiod having 40 μmol/m^2/s light intensity for all cultures was adjusted.

2.2. Establishment of callus cultures

For establishing callus, stem explant was excised from 4 week-old plantlets (in vitro). Explant was inoculated using the optimized protocol [23]. Stem explants (~1.0 cm long) were inoculated on MS media modified with NAA (α-naphthalene acetic acid) 1.0 mg/L, sucrose 30 g/L, agar 8 g/l and pH was adjusted to 5.6 using 2 M NaOH (sodium hydroxide) prior to autoclaving (121 °C, 20 min)and kept at above said conditions for growth. The explant derived calli were subcultured after 4 weeks to ensure 100% callogenesis. The experiment was executed in triplicates. Chemicals used in experimentation were all purchased from Sigma Corporation (USA).

2.3. Cell suspension culture

For cell suspension culture, the 3-weeks old calli (subcultured) were inoculated in Erlenmeyer flasks (250 ml) containing liquid MS media with sucrose (30 g/L) and 1.0 mg/l NAA. Flasks were kept on gyratory shaker (25 ± 2 °C) at constant agitation (120 rpm) in 16/8 h (light/dark) photoperiod with light intensity of 40 μm/m^2/s for 15 days. For inoculum preparation 250 ml Erlenmeyer flasks containing 100 ml Media (each flask) were used.

2.4. Elicitor preparation and treatment

Chitosan $(C6H11NO4)_n$ (deacetylating grade: 70–85%) was used as an elicitor. Chitosan was dissolved in 0.1% acetic acid at 50 °C with continuous stirring for 5 h. For elicitation liquid MS media was prepared having sucrose 30 g/L and NAA 1.0 mg/L. To that media different concentrations (0.1, 0.5, 1.0, 10, 20, 50, 100, 200 and 500 mg/L) of chitosan were added. Prior to autoclaving (121 °C, 20 min), pH was set to 5.6. Experiment was performed in Erlenmeyer flasks (100 ml) containing 40 ml of media. To each flask 10 ml of inoculum (15 days old fine cells) was added. Triplicate flasks were used for each concentration. Cultures were kept on gyratory shaker (25 ± 2 °C) at constant agitation (120 rpm) in 16/8 h (light/dark) photoperiod. MS media containing sucrose 30 g/L and NAA 1.0 mg/l without any elicitor was used as control. Observation of the experiment and tracking data of the growth dynamics and secondary metabolites accumulation was executed with the gap of 5 days interval for 50 days.

2.5. Biomass determination

To determine the fresh weight (FW), respective cell cultures were harvested through filtration using 0.45 μm stainless steel sieve (Sigma), to remove any attached media, cell cultures were gently washed using dH2O. To remove the excess water, cells were gently rubbed on filter paper sheets and cells were weighed for FW. To obtain dry weight (DW) cell were dried in oven (45 °C, 48 h).

2.6. RNA extraction

Total RNA was extracted from 100 mg of liquid nitrogen ground frozen tissues using the Plant GeneJet RNA Purification Kit (Life Technologies) according to supplier instructions. Quantification of RNA was each performed using a fluorometer, with the Quant-iT RNA Assay Kit (Invitrogen) adapted for the Qubit fluorometer, according to the manufacturer's protocol.

2.7. RT-qPCR analysis

Reverse transcription was performed using a First-strand cDNA synthesis kit (Thermo). Quantitative PCR was performed in 96-well plates with a PikoReal real time PCR system (ThermoFisher) using the DyNAmoColorFlash SYBR Green qPCR Kit (ThermoFisher). Each reaction was made in 10 μL (1 μM of each primer pairs, 0.5 μL diluted cDNAs and 2 × SYBR Green mix). All PCR reactions were carried out with the following protocol: 7 min at 95 °C, 40 cycles of 10 s at 95 °C, 10 s at 55 °C and 30 s at 72 °C. The specificity of the amplified product was confirmed for each primer pair, via a melting curve. Data analysis was carried out with Pikoreal software. Three biological replicates and two technical repetitions were performed for each sample. Relative transcript levels were obtained using specific primers (Table S1), designed with Primer3 software, and normalized via the comparative ΔΔCq method using two validated housekeeping reference genes selected by Huis et al. [24]: *LuCYC* encoding for cyclophilin and *LuETIF5 A* encoding for Eukaryotic Translation Initiation Factor 5 A. Results were presented on a heat map format using the MeV software computed with a hierarchical clustering analysis (HCA) representation employing the Euclidean distance as a clustering method with a complete linkage clustering as parameters.

2.8. PAL activity

Soluble proteins were extracted from 1.5 *g* of fresh frozen tissue by homogenization in 3 ml of 0.1 M sodium borate buffer (SBB) pH 8.8 containing 10 mM β-mercaptoethanol and kept on ice for 30 min. After centrifugation (10 min, 16,000 *g*) at 4 °C the supernatant was collected and used in the assay. Protein concentrations were quantified using a Qubit fluorimeter and the Quant-iT Protein Assay Kit (Invitrogen) according to the manufacturer's protocol.

PAL specific activity was assayed spectrophotometrically by monitoring the production of *trans*-cinnamate at 290 nm as previously

described [25]. The reaction mixtures contained 50 lg proteins and 50 mM L-phenylalanine in 5 ml of SBB (pH 8.8) and were incubated at 40 °C.

2.9. CCR and CAD activities

Soluble proteins were extracted by grinding 1.5 *g* of fresh frozen tissue in a pre-chilled mortar containing a cold extraction buffer (0.1 M Tris−HCl pH 7.5, 5% w:v ethylene glycol, 2% w:v polyvinyl polypyrrolidone and 0.1 M *β*-mercaptoethanol). The crude extract was centrifuged (10 min, 16,000 *g*) at 4 °C and the supernatant was used in the assays. Protein concentrations were quantified using a Qubit fluorimeter and the Quant-iT Protein Assay Kit (Invitrogen) according to the manufacturer's protocol.

CCR specific activity was determined spectrophotometrically as described previously [26] using feruloyl-CoA as substrate. Feruloyl-CoA was synthesized according to the optimized protocol reported previously [27].

CAD specific activities were determined spectrophotometrically as described earlier [28]

2.10. Plant extracts preparation

Extracts of cell cultures were prepared following the protocol described previously [29] with slight modifications. Dried cell cultures were grounded finely and mixed with methanol in 1:5 (100 mg in 500 ul). All mixtures were sonicated for 30 min and followed by vortexing for 5 min, this process is repeated twice. These mixtures were then kept on gyratory shaker (25 ± 2 °C) at constant agitation (80 rpm) for 24 h. The sonication and vortexing were repeated and finally the mixtures were centrifuged at 10,000 rpm for 10 min and supernatant was collected and subsequently syringe filtered and stored at 4 °C to be analyzed. For the extraction of lignans and neolignans from cell cultures of flax the protocol described by [4] was followed. Extraction of lyophilized cells was carried out using 80% v/v (20 ml) methanol (aqueous) using ultra sonication (USC1200TH) having 30 kHz frequency for 1 h at 25 ± 2 °C. The mixture was subjected to centrifugation. The supernatant was extracted and evaporated (40 °C) and followed by suspending (4 h at 40 °C) in 0.1 M (1 ml) 4.8 pH buffer (citrate-phosphate). In order to release the aglycones the buffer was equipped with β-glucosidase from almonds (5 units/ml; Sigma Adrich).

2.11. Determination of total phenolic production

Total phenolic content (TPC) was evaluated according to the previously reported protocol [22] using Folin-Ciocalteu (FC) reagent. Twenty microliter of sample extract and 90 µl of FC reagent were mixed and diluted 10x using dH2O followed by incubation for 5 min at 25 ± 2 °C. Sodium carbonate (6%, w/v) was added to the mixture. Absorbance was recorded by Absorbance Microplate Reader (ELx808 BioTek, USA) at 725 nm. In order to plot the calibration curve (R^2 = 0.967), 0-40 µg/ml of gallic acid used as standard. TPC was expressed as equivalents of gallic acid (GAE)/g of dry weight). Eq. (1) was used to calculate the total phenolic production (TPP).

$$Total\ phenolic\ production \frac{mg}{l} = DW\left(\frac{g}{l}\right) \times TPC\left(\frac{mg}{g}\right) \quad (1)$$

TPP was expressed in mg gallic acid/l.

2.12. Quantification of lignans and neolignans by RP-HPLC

The extract was centrifuged for another time and prior to injection the resultant supernatant was filtered (0.45 µm). The quantification of Lignans and neolignans aglycones was carried out using RP-HPLC by Varian liquid chromatographic system equipped with a Varian Prostar 230 pump, a Metachem Degasit, a Varian Prostar 410 autosampler and a Varian Prostar 335 Photodiode Array Detector (PAD) and controlled by Galaxie version 1.9.3.2 software. For separation the method described by [21] was followed using Purospher RP-18 (Merck) column (250 × 4.0 mm i.d.; 5 µm). Calibration curves were used to perform the quantification of Lignans and neolignans. In order to compare the results with published literature easily, results were expressed as mg of glycosides like SDG, LDG, DCG and GGCG equivalent per gram of dry weight. Molecular weight of compounds was used for conversion. Eq. (2) was used to calculate the productivity of lignans and neolignans:

$$Lignans\ and\ neolignans\ productivity \frac{mg}{l} = DW\left(\frac{g}{l}\right) \times accumulation\left(\frac{mg}{g}\right) \quad (2)$$

2.13. Method validation

The quantification was performed with standard calibration curves obtained using five standard dilutions ranging from 50 to 1000 µg/ml. Each standard solution was injected in triplicate. Arithmetic means of each triplicate were calculated. The linear regression equations were carried out by plotting the peak areas against the injected amounts of standard compounds. The linearity was demonstrated by coefficient of determination (R^2). The limits of detection (LOD) and the limits of quantification (LOQ) were determined based on the signal-to-noise ratio (S:N) of approximately 3:1 and 10:1, respectively. Accuracy was evaluated by measuring recovery rates. Dried flax cell cultures were homogenized and separated into two parts of equal mass, one of which was spiked with a known volume of stock solutions. The spiked and non-spiked parts were analyzed by HPLC in triplicate following the procedures described. The recovery rates were calculated according to the following formula:

Recovery rate = (amount in spiked part − amount in non-spiked part) / (spiked amount) x 100.

The method precision and stability were evaluated by determining the intraday and interday variations respectively, which were calculated from data obtained by the repeated injections of standard solutions. The intraday variation was obtained by five replicates in a day and the interday variation was determined by three injections over three continuous days. Retention times and peak areas were assessed. The precision was further checked by measuring the repeatability using five continuous injections of the same extracted sample. The precision was expressed as the relative standard deviation (RSD, %).

2.14. Determination of antioxidant activity

For evaluation of antioxidant activity 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (FRSA) was performed according to the reported protocol [30] with minor alterations. Twenty microliter of sample extract mixed with 180 µl of DPPH (3.2 mg/100 ml methanol) and the resultant mixture was incubated at 25 ± 2 °C for 60 min and followed by the addition of 160 µl of dH_2O. Absorbance was recorded by Absorbance Microplate Reader (ELx808 BioTek, USA) at 517 nm. In order to plot the calibration curve (R^2 = 0.989) methanolic extract of DPPH solution (0.5 ml) was used as standard. The following Eq. (3) was used to calculate the free radical scavenging activity as percentage of discoloration of DPPH.

$$Free\ radical\ scavenging\ activity\ (\%) = 100 \times \left(1 - \frac{Ac}{As}\right) \quad (3)$$

where AC stands for absorbance of the solution when sample extract was mixed at a specific concentration, and AS denotes the absorbance of standard (DPPH solution).

Ferric reducing antioxidant power (FRAP) was measured as described by [31] with some modifications. Briefly, 10 µl of the extracted sample was mixed with 190 µl of FRAP (10 mM TPTZ; 20 mM $FeCl_3$ $6H_2O$ and 300 mM acetate buffer pH3.6; ratio 1:1:10 (v/v/v)). Incubation lasted 15 min at room temperature. Absorbance of the reaction mixture was measured at 630 nm with a BioTek ELX800 Absorbance Microplate Reader. Assays were made in triplicate and antioxidant capacity was expressed as Trolox C equivalent antioxidant capacity (TAEC).

2.15. Statistical analysis

All the experiments were carried out in a synchronized manner and repeated twice. Each treatment was consisted of triplicates. All the mean values were analyzed using Pareto analysis of variance (ANOVA). The significance at $P < 0.05$ was determined by Duncan's multiple range test (DMRT, Windows version 7.5.1, SPSS Inc., Chicago) Graphs were generated using Origin pro (8.5).

3. Results and discussion

3.1. Influence of different concentrations of chitosan on biomass accumulation and (neo) lignans biosynthesis

The effects of different concentrations of chitosan (0.1–500 mg/L) were evaluated on cell cultures of *L. usitatissimum* for biomass accumulation (FW and DW). Data recorded for chitosan-treated (each concentration) and control cell cultures after 30 days. All treatments were also analyzed for its total phenolic content (TPC). Maximum biomass accumulation (both fresh weight (FW) and dry weight (DW)) occurred at 10 mg/l of chitosan (hereafter called CHI-10) as compared to control. Morphological changes induced by different concentrations of chitosan (0.1–500 mg/L) treatments of flax cell suspensions are presented in Fig. 2A. The cell culture treated with 10 mg/l chitosan also presented the highest accumulation of TPC (Table 1) evidencing an activation of phenylpropanoid metabolism.

Table 1
Growth (fresh weights (FW) and dry weights (DW)) and total phenolic concentrations (TPC) in control and chitosan-treated (at different concentration levels) flax cell cultures.

Treatment (mg/l)	FW (g/l)	DW (g/l)	TPC (mg/g DW)
Control	60.35 ± 3.02	5.50 ± 0.07	9.1 ± 0.1
0.1	187.08 ± 5.16	12.55 ± 0.15	10.37 ± 0.13
0.5	130.58 ± 4.39	9.71 ± 0.09	13.9 ± 0.26
1	176.08 ± 4.89	13.10 ± 0.27	14.32 ± 0.29
10	390.66 ± 8.11	16.31 ± 0.33	19.36 ± 0.39
20	193.25 ± 5.05	10.24 ± 0.28	15.34 ± 0.33
100	121.66 ± 3.23	11.32 ± 0.11	13.76 ± 0.27
200	56.33 ± 2.71	5.50 ± 0.08	10.9 ± 0.14
500	35.83 ± 1.19	12.55 ± 0.17	8.1 ± 0.09

Following this first evaluation, to assess the impact of chitosan on phenylpropanoid pathway, transcript accumulation of monolignols as well as lignans and neolignans (i.e., monolignol-derived products) biosynthetic genes by RT-qPCR (Fig. 3) and enzyme activity of key enzymes involved in monolignols biosynthesis (Fig. 4) in flax cell suspensions treated with 0 (control, CTL), 1 (CHI-1), 10 (CHI-10) and 100 (CHI-100) mg/l chitosan were evaluated.

The expression of i) monolignols biosynthetic genes *LuPAL* encoding for a phenylalanine ammonia lyase, *LuCCR* encoding for a cinnamoyl-CoA reductase and *LuCAD* encoding for a cinnamyl alcohol dehydrogenase, previously characterized in flax cell suspension challenging with elicitors [32], ii) lignans biosynthetic genes, encoding for biochemically characterized enzymes, LuDIR5 encoding for a (-)-pinoresinol forming dirigent protein [33,34], *LuPLR1* encoding for a (-)-pinoresinol/(-)-lariciresinol reductase [35,36] and *LuUGT74S1* encoding for a (+)-secoisolariciresinol uridine glycosyltransferase [37], and iii) neolignans biosynthetic genes *LuPCBER* encoding for a phenylcoumaran benzylic ether reductase [35] (Fig. 3A), were monitored after 8 h, 24 h and 48 h after chitosan addition and their relative gene

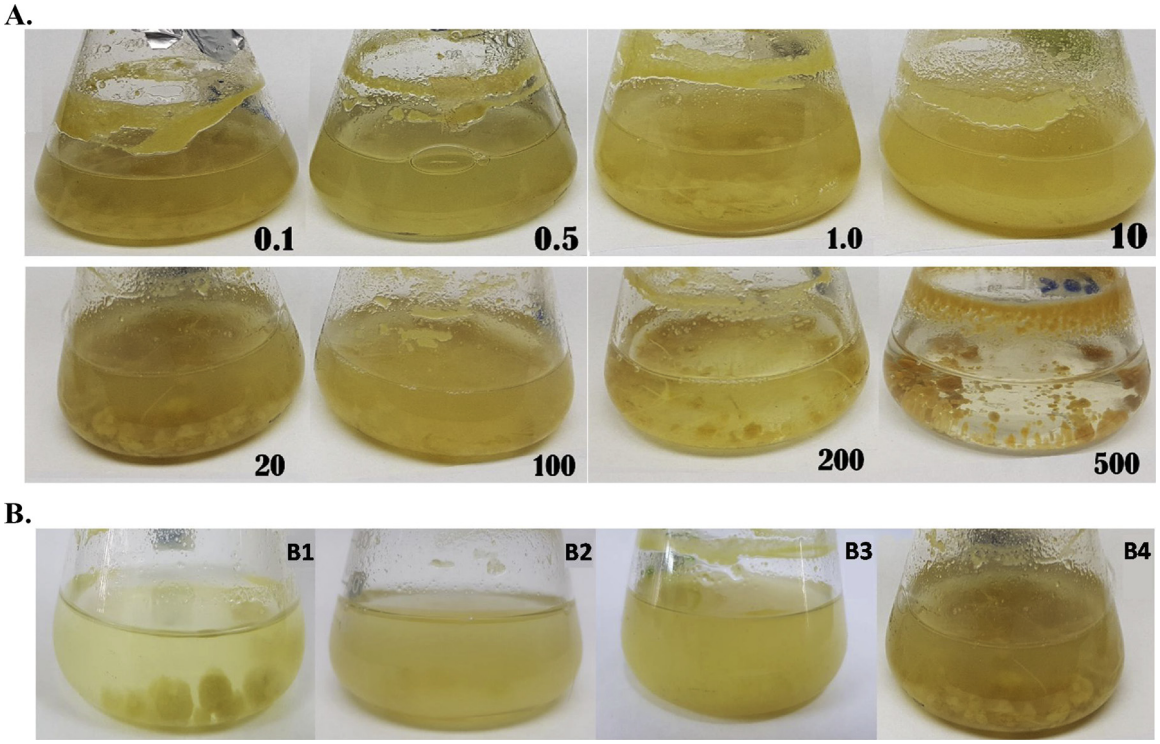

Fig. 2. A. Morphological characteristic of flax cell cultures grown in 0.1–500 mg/L of chitosan at day 30 of culture. B. Morphological characteristic of the different growth phases of flax cell cultures grown in 10 mg/l of chitosan. B1: lag phase. B2: exponential phase. (Log) B3: stationary phase. B4: death phase.

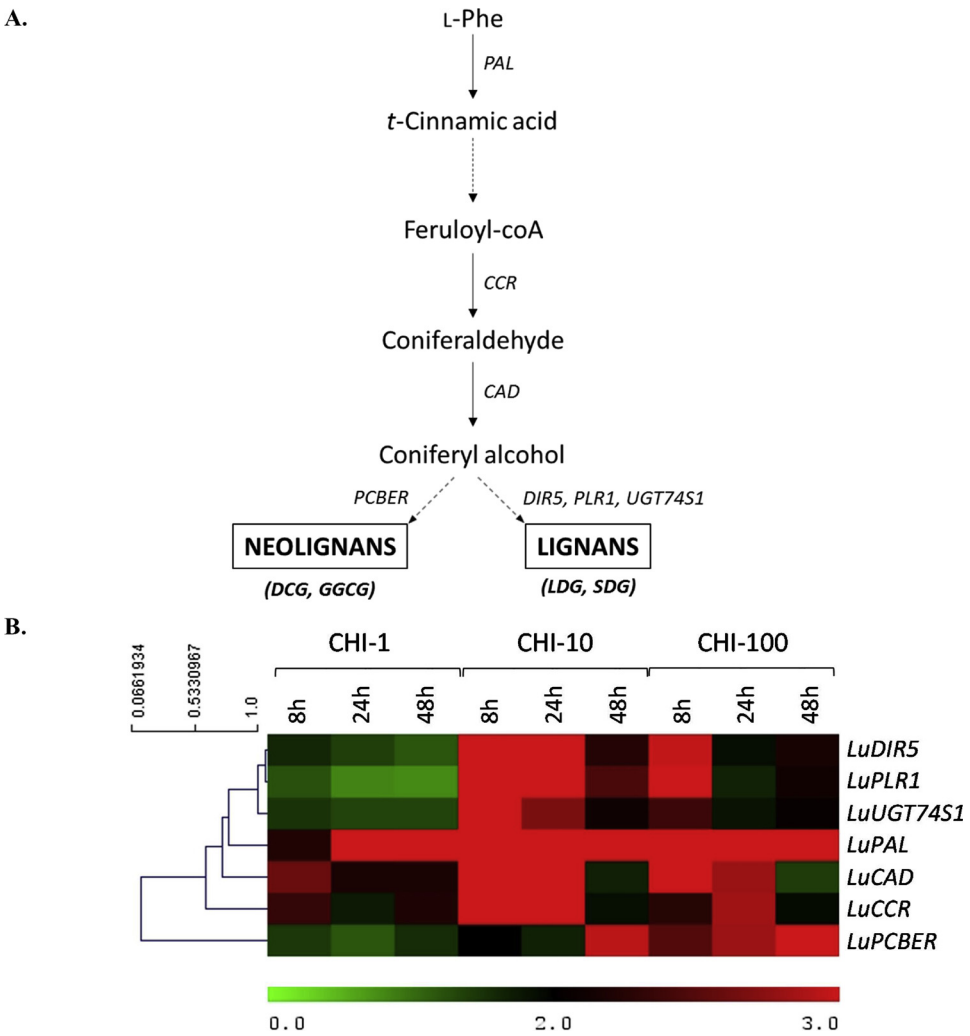

Fig. 3. A. Monolignol and monolignol-derived biosynthetic pathway occuring in flax cell suspension (from Hano et al., 2006a). PAL: phenylalanine ammonia-lyase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl alcohol dehydrogenase; DIR5: dirigent protein 5; PLR1: pinoresinol lariciresinol reductase 1; UGT74S1: secoisolariciresinol uridine glucosyl transferase; PCBER: phenylcoumaran benzylic ether reductase; L-Phe: L-phenylalanine; SDG: secoisolariciresinol diglucoside; LDG: lariciresinol diglucoisde; DCG: dehydrodiconiferyl glucoside; GGCG: guaiacylglycerol-*β*-coniferyl ether diglucoside. B. Normalized relative gene expression profiles in flax cell suspension as determined by RT-qPCR (normalized with cyclophilin (*CYC1*) and Eukaryotic Translation Initiation Factor 5 A (*ETIF5 A*)) following exogenous addition of chitosan at 1 (CHI-1), 10 (CHI-10) and 100 (CHIU-100) mg/l. Results are presented as a clustering classification realized by a hierarchical clustering analysis (HCA) based on the complete linkage Pearson uncentered correlation method performed with MeV.

expression levels (relative to control cell suspension) are presented in Fig. 3B. Here, chitosan treatment triggered the expression of monolignols, lignans and neolignans biosynthetic genes. This gene expression stimulation appeared to be dependent on the chitosan concentration applied with a highest stimulatory effect observed with 10 mg/l chitosan treatment (CHI-10, Fig. 3B). The gene expression kinetic also appeared specific to the metabolic steps thus highlighting the possibility of a coordinated regulation of these metabolic pathways.

PAL, CCR and CAD specific activities were then monitored in control and chitosan-treated cell suspensions during a 48 h period following chitosan addition (Fig. 4). In a good agreement with the gene expression analyses, a rapid, strong and dose-dependent stimulation of PAL activity was observed in all treated cell cultures, reaching a maximum activation 24 h after chitosan addition (Fig. 4A). In the same way, a very similar rapid, transient and dose-dependent activation of both CCR and CAD activities were observed for chitosan-treated cells (Fig. 4B, C). From these enzyme activities data, it appeared that saturation occurred beyond 10 mg/l chitosan addition.

Activation of genes, at both expression and enzyme levels, during the early steps of the phenylpropanoid pathway in response to biotic or abiotic stresses has already been reported in other plant systems. The PAL enzyme catalyzes the entry point of L-phenylalanine into the phenylpropanoid pathway. This enzyme is known to play a crucial role in plant defense mechanisms and is presumably responsible for the increased carbon flux into this pathway leading to an increased biosynthesis of defense/stress-related compounds deriving from this phenylpropanoid pathway [38]. Chitosan induces the enzymatic defensive mechanisms in plants by producing chitinases, pectinases and glucanases enzymes and stimulates plant's immunity which results in enhanced accumulation of biomass while some reports suggest that chitosan is also responsible for enhancing the availability and uptake of water as well as essential nutrients by regulating the osmotic pressure of cells [39,40]. In agreement with our results Mathew & Sankar [41] also reported up to 3.5 times enhancement in biomass accumulation in chitosan treated cell cultures of three species of *Ocimum* i.e. *O. basilicum*, *O. sanctum* and *O. gratissimum*. Similar results were also reported by [42] for *O. basilicum* after application of chitosan. From its polycationic *β*-1,4 linked D-glucosamine polymer chemical structure, chitosan could be consider either as a biotic elicitor or fertilizer as a source of nitrogen and sugars [18].

Here, gene expression analyses and enzyme kinetics, confirmed the trend observed for TPC measured in the corresponding cell suspensions (Table 1). From all these results, 10 mg/l concentration of chitosan was selected for further investigation.

3.2. Effect of chitosan on biosynthesis and productivity of lignans and neolignans

For determination of trends in growth kinetics and biomass accumulation data was monitored periodically at an interval of 5 days for a total of 50 days (Fig. S1). The growth curves of cell cultures of flax grown in 10 mg/l of chitosan exhibited nearly same degree of lag phase (10 days), log phase (exponential phase) starting from day 10 to day 30 comprised of 20 days and followed by 10 days of stationary phase from

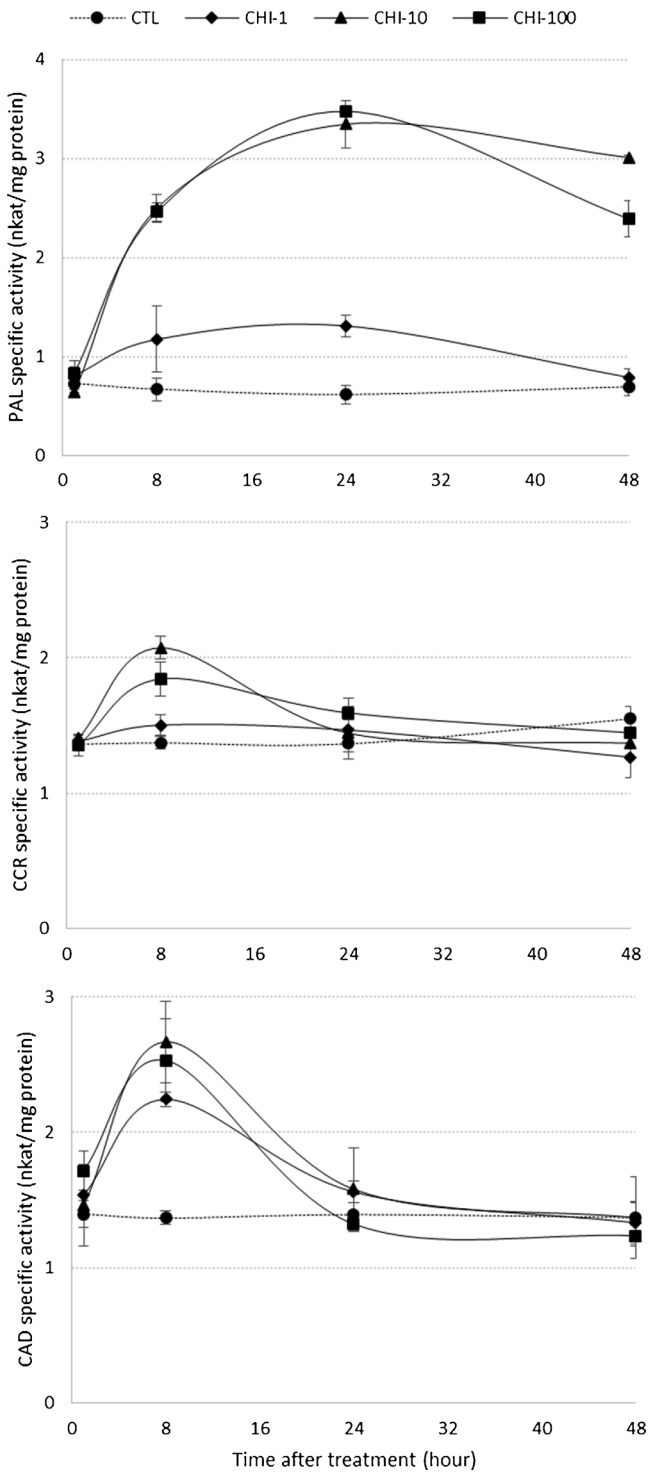

Fig. 4. Time course of specific PAL (A), CCR (B) and CAD (C) activities of the soluble protein fraction of control and chitosan-treated (at 1 (CHI-1), 10 (CHI-10) and 100 (CHIU-100) mg/l) cell suspension cultures. CTL is control cells. Bars represent SE of 3 independent experiments.

day 30 to day 40. From day 40 onwards decline phase was observed (Fig. 2B, Fig. S1).

Chitosan exhibited a positive influence on accumulation of total phenolic content (Fig. S2). Upon analysis it was noted that highest TPC accumulation of 19.36 mg/g DW and a productivity of 315.89 mg/l were recorded at 30th day of inoculation. The probable explanation behind this enhancement is that chitosan upon contact with plant cells induces antioxidant defense mechanism, as a result plant synthesize phenolic compounds to scavenge harmful reactive oxygen species (ROS) [43]. Chitosan also has a possible key role in signaling pathways of biological synthesis of phenolics [44]. The accumulated TPC in Flax cell cultures grown in 10 mg/l of chitosan is 2.1 times (19.36 mg/g DW) higher than control (9.1 mg/g DW) (Fig. 5). Similar findings were reported previously [45] who obtained a 2 fold increase in phenolic content in chitosan elicited adventitious root cultures of *Morinda citrifolia*.

Lignans and neolignans are polyphenols formed via phenylpropanoid metabolic pathway from a common precursor (the monolignol coniferyl alcohol). These polyphenolic compounds are pharmacologically important [46].

To get further insight in their accumulation profile and kinetics, the main lignans (SDG and LDG) and neolignans (DCG and GGCG) accumulated in flax cell cultures were quantified using RP-HPLC method (Figure S3). For this purpose the HPLC-DAD method used to quantify these lignans and neolignans, in the different plant materials analyzed in the present study, was validated in term of precision, accuracy, stability and repeatability. Results of this validation are presented in Table S2. Representation of the peak area and standard concentrations revealed high linear correlations in the range of 50–1000 µg/mL. The linear regression of the 5-point calibration graph showed a R^2-value ranging from 0.9989 for LDG to 0.9998 for DCG, whereas the slope of the standards covering the analytical range varied at most 1% relative standard deviation (RSD) over a four weeks period. In term of limits of detection (LOD, S/N = 3) and limits of quantification (LOQ, S/N = 10), values obtained were 3.4 ng and 10.6 ng for LDG, 2.7 ng and 9.2 ng for SDG, 3.1 ng and 10.0 ng for DCG, and 4.2 ng and 13.4 ng for GGCG, respectively. The determination of the instrumental precision was realized by five injections of the same sample. The chromatographic method used proved its precision (intraday precision) with RSD values ranging from 0.45 (SDG) to 1.25 (LDG). The same sample was injected six times (0, 6, 12, 24, 48 and 72 h after its preparation) in order to evaluate the method stability (interday precision). The small observed values for the RSD, from 0.84 (SDG) to 1.65 (GGCG) confirmed the good stability of the extracted sample. Application of the whole extraction procedure three times to the same batch of material allowed the verification of the high repeatability with RSD values ranging from 0.99 (SDG) to 2.16 (LDG). The separation method accuracy measurement was assessed with standard addition at three concentration levels (50%, 100% and 150%) and a good recovery of the compounds ranging from 97.5% (DCG) to 102.5 (GGCG) was observed.

In current study, lignans (SDG and LDG) and neolignans (DCG and GGCG) were accumulated in their glycosylated forms since aglycones were not detected before *β*-glucosidase hydrolysis (data not shown). The maximum accumulation of lignans and neolignans were observed at day 30 and 35, respectively (Fig. 5). All these current results are in agreement with previously reported results [4,19,20]

Upon evaluation of growth dynamics it was noted that the maximum accumulation of SDG occurred at 35th day (stationary phase) of inoculation (Fig. 5A). Results revealed that maximum accumulation of lariciresinol diglucoside (LDG) was observed at 40th day of inoculation in cell cultures of *L. usitatissimum* (Fig. 5B). The accumulation of LDG in late stationary phase is showing its involvement in alleviating nutrients depletion-induced stress.

Furthermore, the maximum accumulation of dehydrodiconiferyl alcohol glucoside (DCG) was observed at 30th day of inoculation in flax cell cultures (Fig. 5C). Surprisingly, chitosan showed inhibitory effects on the accumulation of guaiacylglycerol-β-coniferyl alcohol ether glucoside (GGCG) (Fig. 5D). This observation will deserve future study in order to understand the partition regulation of the monolignol into these different monolignol-derived products upon chitosan treatment.

LDG and DCG showed growth-associated behavior, maximum accumulation observed with enhanced biomass. However, highest accumulation of SDG in late stationery phase showed that its biosynthesis was growth non-associated and its involvement if ameliorating stresses

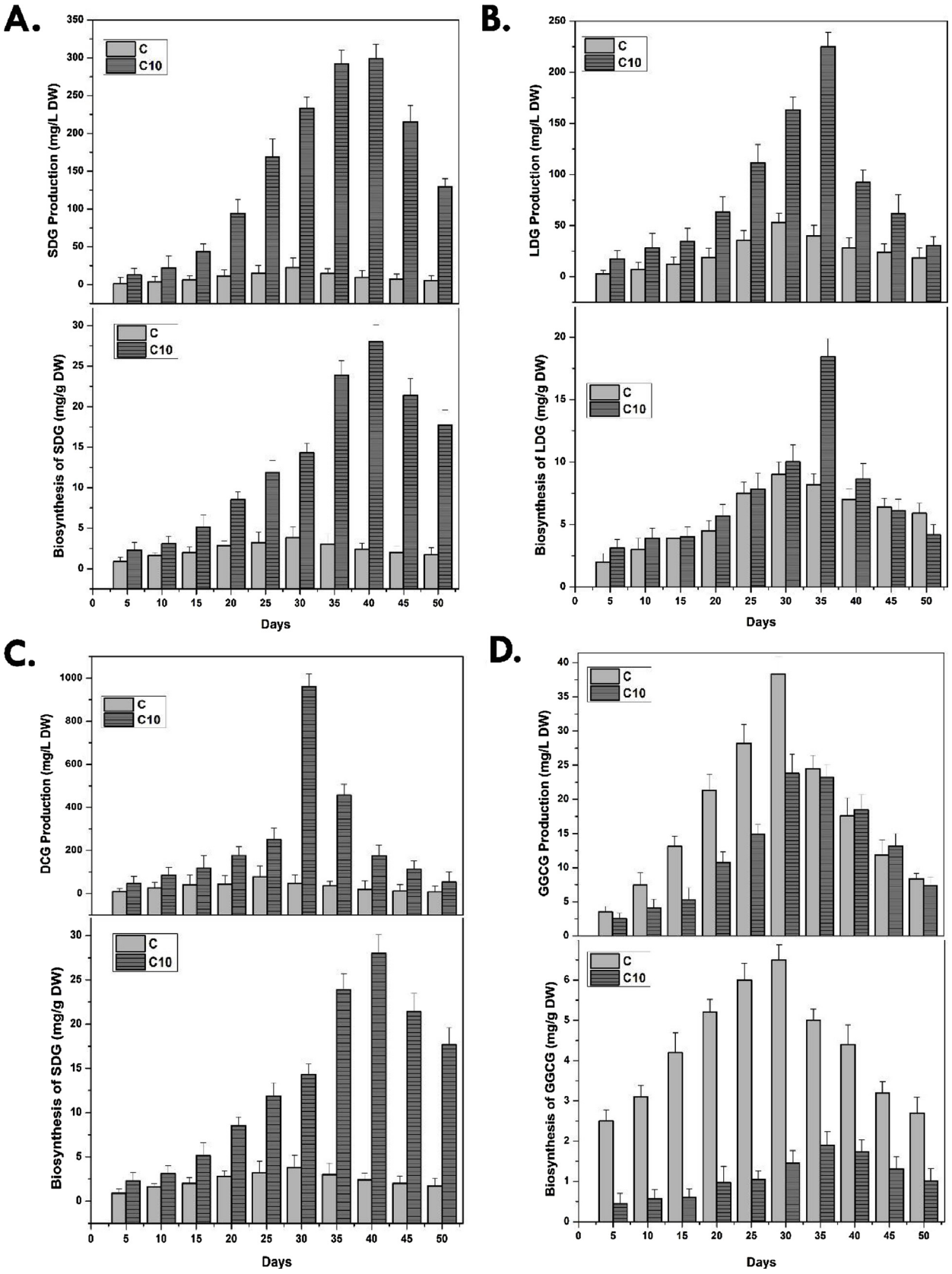

Fig. 5. Biosynthesis and production dynamics of lignans SDG (A) and LDG (B) as well as neolignans DCG (C) and GGCG (D) in chitosan-treated cell cultures of *Linum usitatissimum* (CHI-10) versus control (CTL) flax cell cultures. Values are mean of three triplicates ± SE.

induced by nutrient depletion. Similar enhancing effects of chitosan on accumulation of lignans and neolignans have been reported [43].

From these data, discrepancies can be pointed in our results from the expression levels (Fig. 3), enzyme activities (Fig. 4) and lignans and neolignans production (Fig. 5). First, the expression levels shown in Fig. 3 suggested a very rapid response of gene expression to chitosan induction after 8–24 h of chitosan treatment. On the contrary, the lignans and neolignans production reached the highest levels after 35 days, whereas the expression of related genes appeared to slightly decrease after 48 h of treatment with the 10 mg/l chitosan treatment. This first discrepancy between the accumulations of lignans and neolignans concentration and their biosynthetic gene expression could result from the regulation of key genes involved in monolignol biosynthesis, as it has been reported for the effects of ABA and GA on lignin accumulation by Kim et al. [50] and Zhao and Dixon [51] as well as for the accumulations of lignans and neolignans [52]. For example, CAD and/or COMT genes could be the target of such regulation [50,53] thus modifying the availability of the precursor monomers for lignin or lignans and neolignans biosynthesis. For instance, CAD is of particular interest, since here the CCR and CAD enzyme activities, could also appear not consistent with their corresponding gene expression levels. Indeed, the CCR and CAD enzyme activities reached their maxima 8 h post-treatment and then decreased, whereas their expression remained at very high levels. Nevertheless, we have to keep in mind that CCR and CAD both belong to small multigene families, therefore the enzyme activities measured can result from the action of distinct enzymes encoded by different genes involved in different biosynthetic pathways. Here, we have determined gene expression profile of CCR and CAD genes previously associated with the production of lignans and neolignans in flax cell suspensions [35]. However, other CCR and CAD genes could be associated with the production of lignin as reported by Le Roy et al. [54] and can contribute to the overall enzyme activities measured. The induction of gene expression associated with a decrease in enzyme activity could not only be due to a differential transcriptional regulation within a multigene family, but also result from posttranslational regulation events also known to occur in the phenylpropanoid pathway. This have been, for example, described by Allwood et al. [55] evidencing the implication of phosphorylation event(s) and the involvement of a protein kinase in the posttranslational regulatory mechanism of the PAL enzyme. Moreover, to date no data about the turnover of proteins involved in production of lignans and neolignans are available.

Last, these apparent discrepancies between gene expression levels, enzyme activities and lignans and neolignans production might be due to the fact that the first results provide an indication of the gene expression of the enzyme activity at a precise time whereas metabolite accumulation summarize the effect during the whole duration of the experiment.

Finally, we cannot exclude a possible metabolization of chitosan into a more or less active elicitor compound(s) by flax cells following its addition to the culture medium.

3.3. Impact of chitosan-treatment on antioxidant potential of flax cell culture compared to flax seeds

DPPH assay was conducted to examine the antioxidant potential of the flax cell cultures. It was noted that the cell cultures exhibited highest antioxidant potential (93.6%) at its 30th day of inoculation as compared to control cell cultures (Fig. 6). ROS are normally formed during light dependent processes in plants and can lead to oxidative stress which can be extremely harmful for photosynthetic cells and can damage cellular compounds like membrane lipids, proteins or nucleic acid [47]. Chitosan and its derivatives are involved in accumulation of hydrogen peroxide (H_2O_2) in plants which has a key role in oxidative burst and stimulation of scavenging system of ROS in plants [48,49]. Chitosan is considered to be responsible for alleviation of membrane lipid peroxidation which results in lowering the phytotoxicity in plant

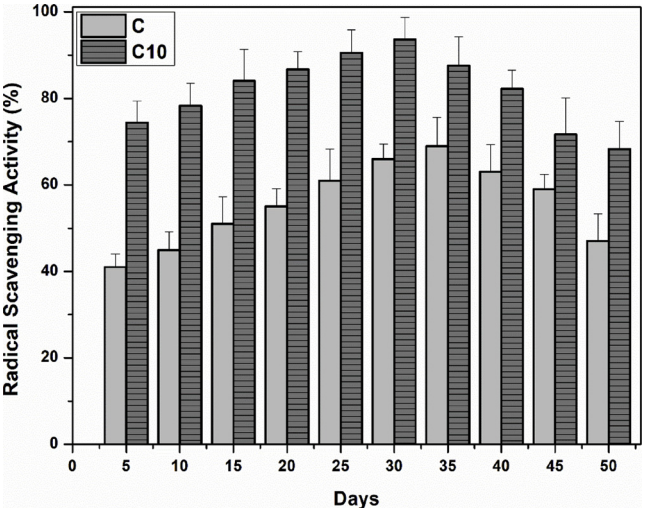

Fig. 6. Antioxidant potential (radical scavenging activity) of cell cultures of *Linum usitatissimum* grown in chitosan (CHI-10) versus control (CTL) cell cultures.

cells by reducing the high chemical oxygen demand. All such antioxidative activities contribute additively to the free radical scavenging capacity of a medicinal plant, therefore, free radical scavenging activity was performed in order to analyze and compare the antioxidant potential of chitosan elicited and control cell cultures of Flax. Results showed that 10 mg/l chitosan efficiently enhanced the free radical scavenging (93.6%) compared to control (69%). Similar results were observed for chitosan treated cell cultures of *Ocimum basilicum* [41].

Last but not least, Table 2 presents the comparison of the accumulation of lignans and neolignans and resulting antioxidant activity of the extracts prepared from control and chitosan-treated flax cell suspensions *vs* wild seeds (from Barbara cultivars known to be highly productive in term of lignans accumulation) [34]; and *LuPLR1* gene silenced transgenic flax [21]. Seeds of wild type flax mainly accumulated SDG whereas seeds of transgenic RNA silencing *PLR1* transgenic flax mainly accumulated neolignans. On the contrary flax cell suspensions accumulated both lignans and neolignans, and chitosan treatment was able to enhanced lignans and neolignans accumulation in this in vitro system. Moreover, flax seeds are cultivated only once a year whereas flax cell suspension constituted a continuous and efficient production system. Last important point, our in vitro production system is non transgenic and therefore could be less controversial in term of public acceptance, safety and usefulness. Altogether these results clearly indicated that flax cell suspension and in particular chitosan treatment are effective production systems of antioxidant lignans and neolignans.

4. Conclusion

In current study, we evaluated the impact of a number of concentrations from 0.1 to 500 mg/l of chitosan in order to elicit the biosynthesis of pharmaceutically important polyphenols i.e. lignans (SDG and LDG) and neolignans (DCG and GGCG) in L. *usitatissimum*cell cultures. Among all, 10 mg/l chitosan proved to be highly effective in the stimulation of gene expressions, enzyme activities and metabolite accumulation of anticancer lignans and neolignans than control cells. Beside, chitosan (10 mg/l) also remarkably enhanced the accumulation of biomass (FW and DW) and antioxidant potential of the flax cells. The productivity of lignans and neolignans as well as antioxidant potential of these chitosan-treated cells were higher than those of flax evidencing the interest of this non transgenic in vitro system. Based on the current findings it is suggested that chitosan treatment of cell cultures of L. *usitatissimum* using chitosan (10 mg/l) for the enhanced productivity of

Table 2
Comparison of lignans and neolignans accumulations and antioxidant activities in flax cell culture (control and chitosan-treated) *vs* seeds (wild type seeds and RNAi-*LuPLR1* transgenic seeds).

	in vitro cell cultures		seeds	
	Control	Chitosan-treated	Wild type (cv Barbara)	RNAi-*LuPLR1* transgenic seeds
SDG[a] (mg/g DW)	4.73 ± 0.22^{B}	23.15 ± 2.04^{A}	20.62 ± 4.71^{A}	0.62 ± 0.15^{C}
LDG[a] (mg/g DW)	8.91 ± 0.57^{B}	18.26 ± 1.07^{A}	ND^{D}	Traces^{C}
DCG[a] (mg/g DW)	17.52 ± 0.84^{B}	56.25 ± 6.35^{A}	ND^{C}	14.22 ± 3.67^{B}
GGCG[a] (mg/g DW)	5.52 ± 0.28^{A}	1.42 ± 0.67^{B}	ND^{D}	Traces^{C}
DPPH (%RSA)[b]	68.36 ± 5.12^{BC}	93.37 ± 8.93^{A}	78.25 ± 11.42^{B}	52.14 ± 10.26^{C}
FRAP (TEAC)[c]	287.63 ± 5.38^{C}	485.28 ± 12.14^{A}	319.34 ± 6.57^{B}	156.45 ± 4.12^{D}

ND: not detected below the LOD; Traces: detected below LOQ (see Table 2); [a] maximum accumulation for control and chitosan-treated flax cell suspension; [b] expressed as percentage of radical scavenging activity measured at day 30 for control and chitosan-treated flax cell suspensions; [c] expressed in mM of Trolox C equivalent antioxidant capacity (TEAC) measured at day 30 for control and chitosan − treated flax cell suspensions; Data are expressed as the mean of n = 4 independent experiments ± standard deviation of the mean and different superscript majuscule letters indicate significant differences between conditions ($P < 0.05$).

anticancer polyphenols is a feasible and promising approach.

Authors contributions

WA performed experiments of cell culture, elicitation with chitosan and analyses of TPC, TFC and antioxidant activity. AZ and MN assisted WA with all of these experiments. BHA conceived idea, supervised research and critically evaluated MS. CH contributed to HPLC analysis, performed gene expression analysis and enzymatic measurements and contributed to the conception, writing and critical reading of the manuscript. LG and SD performed HPLC. SR and JD contributed to enzymatic measurements and HPLC quantification. NGG discussed research and critically reviewed the manuscript.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2018.12.025.

References

[1] H. Helbaek, Plant Collecting, Dry-Farming, and Irrigation Agriculture in Prehistoric Deh Luran, (1968).
[2] V. Zeist, Paleobotanical results of the 1970 season at Cayonu, Turkey, Helinium 12 (1) (1972) 3–19.
[3] N.D. Westcott, A.D. Muir, Chemical studies on the constituents of Linum spp, Flax, Genus Linum (2003) 55–73.
[4] S. Anjum, B.H. Abbasi, J. Doussot, A. Favre-Réguillon, C. Hano, Effects of photoperiod regimes and ultraviolet-C radiations on biosynthesis of industrially important lignans and neolignans in cell cultures of Linum usitatissimum L.(Flax), J. Photochem. Photobiol. B, Biol. 167 (2017) 216–227.
[5] R. Haworth, The chemistry of the lignan group of natural products, J. Chem. Soc. (Resumed) (1942) 448–456.
[6] Z.-Q. Xia, M.A. Costa, J. Proctor, L.B. Davin, N.G. Lewis, Dirigent-mediated podophyllotoxin biosynthesis in Linum flavum and Podophyllum peltatum, Phytochemistry 55 (6) (2000) 537–549.
[7] O. Gottlieb, Neolignans, Fortschritte Der Chemie Organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products, Springer, 1978, pp. 1–72.
[8] R.B. Teponno, S. Kusari, M. Spiteller, Recent advances in research on lignans and neolignans, Nat. Prod. Rep. 33 (9) (2016) 1044–1092.
[9] M. Gordaliza, P. Garcıa, J.M. Del Corral, M. Castro, M. Gomez-Zurita, Podophyllotoxin: distribution, sources, applications and new cytotoxic derivatives, Toxicon 44 (4) (2004) 441–459.
[10] M.E. Martin, M. Haourigui, C. Pelissero, C. Benassayag, E.A. Nunez, Interactions between phytoestrogens and human sex steroid binding protein, Life Sci. 58 (5) (1995) 429–436.
[11] D. Li, J.A. Yee, L.U. Thompson, L. Yan, Dietary supplementation with secoisolariciresinol diglycoside (SDG) reduces experimental metastasis of melanoma cells in mice, Cancer Lett. 142 (1) (1999) 91–96.
[12] W. Demark-Wahnefried, D.T. Price, T.J. Polascik, C.N. Robertson, E.E. Anderson, D.F. Paulson, P.J. Walther, M. Gannon, R.T. Vollmer, Pilot study of dietary fat restriction and flaxseed supplementation in men with prostate cancer before surgery: exploring the effects on hormonal levels, prostate-specific antigen, and histopathologic features, Urology 58 (1) (2001) 47–52.
[13] N. Danbara, T. Yuri, M. Tsujita-Kyutoku, R. Tsukamoto, N. Uehara, A. Tsubura, Enterolactone induces apoptosis and inhibits growth of Colo 201 human colon cancer cells both in vitro and in vivo, Anticancer Res. 25 (3B) (2005) 2269–2276.
[14] S.C. Cunnane, S. Ganguli, C. Menard, A.C. Liede, M.J. Hamadeh, Z.-Y. Chen, T.M. Wolever, D.J. Jenkins, High α-linolenic acid flaxseed (Linum usitatissimum): some nutritional properties in humans, Br. J. Nutr. 69 (2) (1993) 443–453.
[15] D. Jenkins, C.W. Kendall, E. Vidgen, S. Agarwal, A.V. Rao, R.S. Rosenberg, E.P. Diamandis, R. Novokmet, C.C. Mehling, T. Perera, L.C. Griffin, S.C. Cunnane, Health aspects of partially defatted flaxseed, including effects on serum lipids, oxidative measures, and ex vivo androgen and progestin activity: a controlled crossover trial, Am. J. Clin. Nutr. 69 (1999) 395–402.
[16] E. Skrzypczak-Pietraszek, J. Słota, J. Pietraszek, The influence of L-phenylalanine, methyl jasmonate and sucrose concentration on the accumulation of phenolic acids in Exacum affine Balf. f. ex Regel shoot culture, Acta Biochim. Pol. 61 (1) (2014).
[17] H.N. Murthy, E.-J. Lee, K.-Y. Paek, Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation, Plant Cell Tissue Org. Cult. (PCTOC) 118 (1) (2014) 1–16.
[18] S. Hirano, N. Nagao, Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens, Agric. Biol. Chem. 53 (11) (1989) 3065–3066.
[19] N. Benhamou, P. Lafontaine, M. Nicole, Induction of systemic resistance to Fusarium crown and root rot in tomato plants by seed treatment with chitosan, Phytopathology 84 (12) (1994) 1432–1444.
[20] R. Cuero, E. Duffus, G. Osuji, R. Pettit, Aflatoxin control in preharvest maize: effects of chitosan and two microbial agents, J. Agric. Sci. 117 (2) (1991) 165–169.
[21] S. Renouard, M.-A. Tribalatc, F. Lamblin, G. Mongelard, O. Fliniaux, C. Corbin, D. Marosevic, S. Pilard, H. Demailly, L. Gutierrez, RNAi-mediated pinoresinol lariciresinol reductase gene silencing in flax (Linum usitatissimum L.) seed coat: consequences on lignans and neolignans accumulation, J. Plant Physiol. 171 (15) (2014) 1372–1377.
[22] B.H. Abbasi, S. Anjum, C. Hano, Differential effects of in vitro cultures of Linum usitatissimum L.(Flax) on biosynthesis, stability, antibacterial and antileishmanial

activities of zinc oxide nanoparticles: a mechanistic approach, RSC Adv. 7 (26) (2017) 15931–15943.
[23] S. Anjum, B.H. Abbasi, C. Hano, Trends in accumulation of pharmacologically important antioxidant-secondary metabolites in callus cultures of Linum usitatissimum L, Plant Cell Tissue Org. Cult. (PCTOC) 129 (1) (2017) 73–87.
[24] T. Koressaar, M. Remm, Enhancements and modifications of primer design program Primer3, Bioinformatics 23 (10) (2007) 1289–1291.
[25] D.C. Dunn, L.W. Duncan, J.T. Romeo, Changes in arginine, pal activity, and nematode behavior in salinity-stressed citrus in honour of Professor GH Neil Towers 75th birthday, Phytochemistry 49 (2) (1998) 413–417.
[26] D. Goffner, M.M. Campbell, C. Campargue, M. Clastre, G. Borderies, A. Boudet, A.M. Boudet, Purification and characterization of cinnamoyl-coenzyme A: NADP oxidoreductase in Eucalyptus gunnii, Plant Physiol. 106 (2) (1994) 625–632.
[27] T. Beuerle, E. Pichersky, Enzymatic synthesis and purification of aromatic coenzyme A esters, Anal. Biochem. 302 (2) (2002) 305–312.
[28] S.W. Hawkins, A.M. Boudet, Purification and characterization of cinnamyl alcohol dehydrogenase isoforms from the periderm of Eucalyptus gunnii Hook, Plant Physiol. 104 (1) (1994) 75–84.
[29] M. Ali, B.H. Abbasi, Production of commercially important secondary metabolites and antioxidant activity in cell suspension cultures of Artemisia absinthium L, Ind. Crops Prod. 49 (2013) 400–406.
[30] H. Fazal, B.H. Abbasi, N. Ahmad, M. Ali, S. Ali, Sucrose induced osmotic stress and photoperiod regimes enhanced the biomass and production of antioxidant secondary metabolites in shake-flask suspension cultures of Prunella vulgaris L, Plant Cell Tissue Org. Cult. (PCTOC) 124 (3) (2016) 573–581.
[31] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1) (1996) 70–76.
[32] C. Hano, I. Martin, O. Fliniaux, B. Legrand, L. Gutierrez, R. Arroo, F. Mesnard, F. Lamblin, E. Lainé, Pinoresinol–lariciresinol reductase gene expression and secoisolariciresinol diglucoside accumulation in developing flax (Linum usitatissimum) seeds, Planta 224 (6) (2006) 1291–1301.
[33] D.S. Dalisay, K.W. Kim, C. Lee, H. Yang, O. Rübel, B.P. Bowen, L.B. Davin, N.G. Lewis, Dirigent protein-mediated lignan and cyanogenic glucoside formation in flax seed: integrated omics and MALDI mass spectrometry imaging, J. Nat. Prod. 78 (6) (2015) 1231–1242.
[34] C. Corbin, S. Drouet, L. Markulin, D. Auguin, É. Lainé, L.B. Davin, J.R. Cort, N.G. Lewis, C. Hano, A genome-wide analysis of the flax (Linum usitatissimum L.) dirigent protein family: from gene identification and evolution to differential regulation, Plant Mol. Biol. (2018) 1–29.
[35] C. Hano, M. Addi, L. Bensaddek, D. Crônier, S. Baltora-Rosset, J. Doussot, S. Maury, F. Mesnard, B. Chabbert, S. Hawkins, Differential accumulation of monolignol-derived compounds in elicited flax (Linum usitatissimum) cell suspension cultures, Planta 223 (5) (2006) 975–989.
[36] C. Von Heimendahl, K.M. Scha fer, P. Eklund, R. Sjo holm, T.J. Schmidt, E. Fuss, Pinoresinol-lariciresinol reductases with different stereospecificity from Linum album and Linum usitatissimum, Phytochemistry 66 (2005) 1254–1263.
[37] K. Ghose, K. Selvaraj, J. McCallum, C.W. Kirby, M. Sweeney-Nixon, S.J. Cloutier, M. Deyholos, R. Datla, B. Fofana, Identification and functional characterization of a flax UDP-glycosyltransferase glucosylating secoisolariciresinol (SECO) into secoisolariciresinol monoglucoside (SMG) and diglucoside (SDG), BMC Plant Biol. 14 (1) (2014) 82.
[38] R.A. Dixon, N.L. Paiva, Stress-induced phenylpropanoid metabolism, Plant Cell 7 (7) (1995) 1085.
[39] S. Bautista-Baños, M. Hernández-López, E. Bosquez-Molina, C. Wilson, Effects of chitosan and plant extracts on growth of Colletotrichum gloeosporioides, anthracnose levels and quality of papaya fruit, Crop. Prot. 22 (9) (2003) 1087–1092.
[40] N.Q. Hien, Radiation degradation of chitosan and some biological effects, Radiat. Process. Polysaccharides 1422 (2004) 67.
[41] R. Mathew, P.D. Sankar, Effect of methyl jasmonate and chitosan on growth characteristics of Ocimum basilicum L., Ocimum sanctum L. and Ocimum gratissimum L. cell suspension cultures, Afr. J. Biotechnol. 11 (21) (2012) 4759.
[42] H.-J. Kim, F. Chen, X. Wang, N.C. Rajapakse, Effect of chitosan on the biological properties of sweet basil (Ocimum basilicum L.), J. Agric. Food Chem. 53 (9) (2005) 3696–3701.
[43] S. Esmaeilzadeh Bahabadi, M. Sharifi, J. Murata, H. Satake, The effect of chitosan and chitin oligomers on gene expression and lignans production in Linum album cell culture, Journal of Medicinal Plants 1 (2014) 53–56.
[44] R. Dixon, M. Harrison, C. Lamb, Early events in the activation of plant defense responses, Annu. Rev. Phytopathol. 32 (1) (1994) 479–501.
[45] M.A. Baque, M.H.K. Shiragi, E.-J. Lee, K.-Y. Paek, Elicitor effect of chitosan and pectin on the biosynthesis of anthraquinones, phenolics and flavonoids in adventitious root suspension cultures of'Morinda citrifolia'(L.), Aust. J. Crop Sci. 6 (9) (2012) 1349.
[46] T.J. Schmidt, M. Klaes, J. Sendker, Lignans in seeds of Linum species, Phytochemistry 82 (2012) 89–99.
[47] C.H. Foyer, M. Lelandais, K.J. Kunert, Photooxidative stress in plants, Physiol. Plant. 92 (4) (1994) 696–717.
[48] G.K. Agrawal, R. Rakwal, S. Tamogami, M. Yonekura, A. Kubo, H. Saji, Chitosan activates defense/stress response (s) in the leaves of Oryza sativa seedlings, Plant Physiol. Biochem. 40 (12) (2002) 1061–1069.
[49] W. Lin, X. Hu, W. Zhang, W.J. Rogers, W. Cai, Hydrogen peroxide mediates defence responses induced by chitosans of different molecular weights in rice, J. Plant Physiol. 162 (8) (2005) 937–944.
[50] Y.-H. Kim, J.M. Bae, G.-H. Huh, Transcriptional regulation of the cinnamyl alcohol dehydrogenase gene from sweetpotato in response to plant developmental stage and environmental stress, Plant Cell Rep. 29 (7) (2010) 779–791.
[51] Q. Zhao, R.A. Dixon, Transcriptional networks for lignin biosynthesis: more complex than we thought, Trends Plant Sci. 16 (4) (2011) 227–233.
[52] C. Corbin, S. Renouard, T. Lopez, F. Lamblin, E. Lainé, C. Hano, Identification and characterization of cis-acting elements involved in the regulation of ABA-and/or GA-mediated LuPLR1 gene expression and lignan biosynthesis in flax (Linum usitatissimum L.) cell cultures, J. Plant Physiol. 170 (5) (2013) 516–522.
[53] P.G. Mohr, D.M. Cahill, Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with Pseudomonas syringae pv. tomato, Funct. Integr. Genom. 7 (3) (2007) 181–191.
[54] J. Le Roy, A.-S. Blervacq, A. Créach, B. Huss, S. Hawkins, G. Neutelings, Spatial regulation of monolignol biosynthesis and laccase genes control developmental and stress-related lignin in flax, BMC Plant Biol. 17 (1) (2017) 124.
[55] E.G. Allwood, D.R. Davies, C. Gerrish, B.E. Ellis, G.P. Bolwell, Phosphorylation of phenylalanine ammonia-lyase: evidence for a novel protein kinase and identification of the phosphorylated residue, FEBS Lett. 457 (1) (1999) 47–52.