Salicylic acid-enhanced biosynthesis of pharmacologically important lignans and neo lignans in cell suspension culture of *Linum usitatissimum* L

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Linum usitatissimum L. (flax) is a perennial herb with magnitude of medicinal and commercial applications. In the present study, we investigated the effects of salicylic acid (SA) on biosynthesis of lignans (secoisolariciresinol diglucoside (SDG) and lariciresinol diglucoside (LDG)) and neolignans (dehydrodiconiferyl alcohol glucoside (DCG) and guaiacylglycerol- β -coniferyl alcohol ether glucoside (GGCG)) in cell cultures of flax. Moderate concentration of SA (50 μ M) enhanced biomass accumulation (10.98 g/L dry weight (DW)), total phenolic content (37.81 mg/g DW), and antioxidant potential (87.23%) to two-fold than their respective controls after 72 h of exposure. However, higher levels of total flavonoid content (5.32 mg/g DW) were noted after 48 h of exposure to 50 μ M of SA. HPLC analyses revealed that 50 μ M SA, significantly enhanced biosynthesis of SDG (7.95 mg/g DW), LDG (7.52 mg/g DW), DCG (54.90 mg/g DW), and GGCG (16.78 mg/g DW), which was almost 2.7, 1.8, 3.88, and 3.98 fold higher than their respective controls after 72 h of exposure time, respectively. These results indicated that moderate concentrations of SA had significant effects on biosynthesis and productivity of lignans and neolignans in cell culture of *L. usitatissimum*.

KEYWORDS

antioxidant, HPLC, lignans, *Linum usitatissimum*, neo-lignans, salicylic acid

1 | INTRODUCTION

Linum usitatissimum L., most commonly known as Flax, is an important medicinal herb belonging to the family Linaceae [1]. The genus encompasses more than 100 species distributed all around the sphere [2]. It is one of the oldest cultivated species with great number of health and industrial

applications [3]. Besides its medicinal applications, it is also used in production of dairy goods, salads, and drinks [1,3,4]. Flax has also been used for decades in traditional therapies to treat pulmonary complications, boost stamina and memory [3]. Recently, anticancer, antimicrobial, anti-diabetic, antioxidant, and anti-inflammatory activities of flax have been explored [5,6]. These therapeutic properties are credited to the biologically active ingredients (BAIs) biosynthesized in flax [3,7]. Lignans and neo lignans are the major BAIs detected in flax [5,8,9]. When flax is consumed by mammals, these BAIs are metabolized into another class of compounds known as phytoestrogens [10]. These phytoestrogens are reported to have an excellent anti-cancer potential against hormone related cancers [10–13].

Abbreviations: BAIs, biologically active ingredients; DCG, dehydrodiconiferyl alcohol glucoside; DW, dry weight; GGCG, guaiacylglycerol- β -coniferyl alcohol ether glucoside; LDG, lariciresinol diglucoside; NAA, α -naphthalene acetic acid; SA, salicylic acid; SDG, secoisolariciresinol diglucoside; TFC, total flavonoid content; TPC, total phenolic content

The biosynthesis of these BAIs in wild grown plants is governed by several environmental factors [14–16]. To overcome these issues, an alternative platform of plant in vitro culture technology was established and evaluated [17]. This platform is offering several advantages over conventional production practices of these plant species [18]. Plant cell culture is widely acknowledged and accepted for biosynthesis of plant derived-BAIs at commercially feasible levels [19]. However, several strategies have been developed to enhance biosynthesis of these BAIs in plant cell cultures to make the production process cost efficient. Elicitation of BAIs with several biotic and abiotic compounds/products is a famous strategy to date [20–22].

Salicylic acid (SA) is a potent plant signaling molecule involved in growth responses and providing resistance against pathogens [23,24]. SA induces defense mechanism via production of compounds involved in defense [25]. When applied exogenously, it mimics the pathogen interaction and prompt the expression of genes and enzymes involved in biosynthesis and catalyzing of phenylpropanoid metabolites [24,26]. SA as an abiotic elicitor has been widely used to enhance the BAIs in plants cell culture systems [26–28]. To date, no report is available on elicitation of lignans and neolignans in Flax cell cultures using SA. Hence the current study was aimed to investigate effects of SA on growth dynamics, antioxidant potential, and accumulation of secondary metabolites including total phenolic, flavonoids, and polyphenols (lignans and neolignans).

2 | MATERIALS AND METHODS

2.1 | Seed germination and callus culture establishment

Seeds were surface sterilized by method of [29] with minor modifications and placed on Murashige and Skoog 0 medium supplemented with 0.8% agar and 3% sucrose. Callus culture was established as previously optimized [29]. A 21 days old seedling stem explant was placed on Murashige and Skoog media fortified with 1 mg/L α -Naphthalene acetic acid, 3% sucrose, and 0.8% agar. Sub culturing was performed on same media after each 21 days. For establishment of suspension culture, approximately 1 g of fresh calli was transferred to 100 mL liquid media supplemented with same media ingredients excluding agar. Upon successful establishment of suspension culture, 5 mL of suspension inoculum was added to 40 mL media and all flasks were transferred to gyratory shaker at 100 rpm placed in optimum growth chamber.

PRACTICAL APPLICATION

Lignans and neolignans are poly-phenolic compounds, biosynthesized in flax (*Linum usitatissimum* L.). These compounds have been widely acknowledged due to their anti-cancer, anti-diabetic, and antimicrobial activities. Due to lack of proper cultivation practices, these plants and metabolites are facing serious threats. An alternative approach of plant in vitro technology has opened up new dimensions to address these challenges. Therefore, plant cell culture of *Linum usitatissimum* was established for biosynthesis of these Lignans and neolignans. Elicitation strategy was devised to further enhance biosynthetic potential of these cell cultures. Salicylic acid has proven its role and stimulated biosynthesis of these lignans and neolignans to feasible production levels in these cell lines. This strategy is cost efficient and reliable for production of lignans and neolignans in flax cell cultures.

2.2 | Elicitor preparation and treatment

SA stock solution was prepared in water according to protocol of [26]. Different concentrations of SA (0, 25, 50, 100, and 200 μ M) were added into exponentially growing flax cell (day 25). For control treatment, same amount of water was added. Each treatment was harvested after 12, 24, 48, 72, and 96 h post elicitation.

2.3 | Analytical scheme

Cell extraction was made with slight modifications in the protocol of [21]. About 200 mg of dry sample was mixed with 0.5 mL HPLC grade methanol, each dilution was placed on a shaker, left overnight for 24 h at room temperature. Later each dilution was sonicated, vortexed, and finally centrifuged at 10000 rpm. Supernatant was stored at 4°C for further analysis. Total phenolic content (TPC) was measured using method of [30]. Briefly 90 μ L of Folin-Ciocalteu (FC) reagent was added to 20 μ L of each sample, later 90 μ L of sodium carbonate was added. Gallic acid was used as control. The absorbance was measured at 630 nm using a 96 micro plate reader. The protocol of [31], was used for quantification of total flavonoid content (TFC). Briefly 10 μ L of potassium acetate was mixed with 20 μ L of each sample; later 10 μ L of aluminum chloride and 160 μ L of distilled water was added and placed for 30 min at room temperature. Quercetin was used as control. The absorbance was

measured at 415 nm using a 96 micro plate reader. Free radical scavenging potential was determined with slight modifications in the protocol of [32]. Briefly, 180 μL of DPPH solution was added to 20 μL of each sample. The mixtures were placed for 1 h in dark at room conditions. The absorbance was checked at 517 nm using a 96 micro plate reader. To estimate FRSA following equation was used:

$$\% \text{ scavenging DPPH free radical} = 100 \times (1 - \text{AE}/\text{AD})$$

where AE represents absorbance of the dilutions at 517 nm and AD indicates the absorbance of the DPPH pure stock solution.

2.4 | HPLC analysis

Lignans and neolignans were extracted by the protocols of [14,33]. Briefly, 100 mg of dried extracts were placed in 80% methanol (v/v). Later each dilution was sonicated and incubated at 25°C for 1 h. Once sonicated, each dilution was centrifuged and supernatant was subjected to 40°C to evaporate any methanolic residue. Later, 1 mL of 0.1 M citrate-phosphate (pH 4.8) buffer containing 5 unit/mL of β -glucosidase (Sigma) was added to release aglycone residues. The mixture was then allowed to pass through (0.45 μm) filter paper prior to HPLC injection. RP-HPLC equipped with Varian Prostar 230 pump, Metachem Degasit, Varian Prostar 410 auto sampler, and Varian Prostar 335 Photodiode Array Detector (PAD) was used for the quantification of lignans and neolignans. Online system equipped with Galaxie version 1.9.3.2 software was used for identification. Purospher (Merck) RP-18 column (250 $\times$ 4.0 mm id; 5 μm) maintained at 40°C was used for the separation [34]. For confirmation of relative proportion of aglycones vs glycosides residues, the same extract was investigated without enzymatic treatment. Lignans and neolignans were measured by using the calibration curves and the results are stated as milligram of glucoside (secoisolariciresinol diglucoside (SDG), lariciresinol diglucoside (LDG), dehydrodiconiferyl alcohol glucoside (DCG), and guaiacylglycerol- β -coniferyl alcohol ether glucoside (GGCG)) equivalent to per gram dry weight (DW). The results were then matched with available data and molecular weight of the particular compounds was used for each compound.

2.5 | Statistical analysis

All the experiments were accomplished in triplicates. Graph Pad Prism Windows, v5.0 was used for statistical analysis and Origin 8.5 for drawing graphs. The data were evaluated as

FIGURE 1 Time course growth of biomass and polyphenol accumulation in cell cultures of flax. Values indicates mean $\pm$ SE

means followed by one-way ANOVA (Tukey's multiple comparisons test, $P < 0.05$).

3 | RESULTS AND DISCUSSION

3.1 | Time course of growth and polyphenol accumulation

The kinetics of biomass accumulation and biosynthesis of polyphenols (lignans and neolignans) in flax cell culture is shown in Figure 1. Flax cell cultures exhibited a shorter lag phase of 10 days while a prolonged log phase from day 20 to 30. Later, this stage decline was noted indicating end of log phase. The highest biomass accumulation was noted in log phase on 30th day. Similar trend was also noted in accumulation of lignans and neolignans in cell culture of flax reaching maximum accumulation on 30th day (Figure 1). Based on kinetics of growth and polyphenol accumulation, flax cell cultures were treated with SA on 25th day for 12, 24, 48, 72, and 96 h.

3.2 | Biomass accumulation

The biomass accumulation DW in flax cell cultures was examined after exposure to different concentrations of SA on 25th day of culturing. Results showed that no significant changes were noted in growth earlier but after 48 h a significant surge was noted which declined with the passage of time. Among the tested concentrations, optimal biomass accumulation (10.98 g/L DW) was noted upon treatment of 50 μM SA for 72 h, which was almost double as compared to control (10.1 g/L DW) (Figure 2). The lowest value was recorded with application of 200 μM SA. The biomass accumulation in flax cell cultures may be ascribed to enhanced uptake of vital nutrients and metabolism yielding high biomass [35]. Our findings are also in agreement with previous report of [26], where they have observed positive effects of SA on growth of *Saussurea medusa* cell cultures. Our results are also in harmony with report of [36], where they found that high

FIGURE 2 Effect of SA on accumulation of biomass DW in flax cell cultures. Values indicates mean $\pm$ SE. Columns with identical letters are not significantly different ($P < 0.05$)

concentration of SA inhibited growth of *Panax ginseng* cell cultures.

3.3 | Biosynthesis of TPC and TFC

Phenolic and flavonoids are low molecular weight compounds playing vital role in plant physiology and development [37, 38]. In the present study, SA considerably stimulated phenolic and flavonoids biosynthesis in cell cultures of flax. TPC and TFC biosynthesis were dosage dependent and increased with the exposure time of elicitation. The highest accumulation of TPC (37.8 mg/g DW) was observed in cells exposed to 50 μ M SA for 72 h, which was almost 1.8-fold higher than control (21 mg/g DW) (Figure 3A). A significant increase was also noted in cells exposed to 100 μ M SA for 72 h. Subsequently, significant decline was noted in TPC, indicating the switching of culture to next phase of growth. The lowest was noted with application of 200 μ M SA. In the literature, the SA has been reported to have encouraging effects on

phenolic contents [36,39,40]. The enhanced accumulation of TPC might be attributed to the stimulation of PAL activities and metabolic pathways [24]. Yielding, a cascade of high branch phenols are produced [40,41].

Unlikely TPC, TFC biosynthesis was started to increase earlier at 24 h, and reached optimum after 48 h exposure. Maximum TFC (5.23 mg/g DW) was recorded with application of 50 μ M SA with 1.3-fold increase than control (3.82 mg/g DW) (Figure 3B). A significant decline was noted later at this point. These results indicate that SA showed positive impact on biosynthesis of TFC. Similarly, some researchers have reported positive impact of SA on flavonoid biosynthesis in various plant species which endorse our results [26,36,42,43]. The accumulation of flavonoids in these studies were related to high expression of genes and enzymes involved in biosynthesis of flavonoids which might be in our case here as well.

3.4 | Antioxidant activities

Plants in stress conditions produce huge amount of reactive oxygen species which in turn harm vital organic molecules, thus limiting plant growth and development [22]. However, plants are blessed with a defense mechanism which neutralizes the reactive oxygen species mediated harmful effects via low molecular weight antioxidants substrates along with antioxidative enzyme system [44,45]. In our study, the antioxidants show enhancement in accumulation after 24 h of exposure to SA. The highest Radical scavenging activity (RSA) was noted on exposure of 50 μ M SA, which was almost double than control. Similarly, the lowest was recorded with the application of 200 μ M SA (Figure 4). SA protects cells from any sort of injury in cells by enhancing the expression of antioxidant enzymes involved in defense [46]. Flax various metabolites i.e., lignans and neolignans have been acknowledged to have excellent antioxidant potential [47]. Thus

FIGURE 3 (A) Effect of SA on biosynthesis of TPC in flax cell cultures. Values indicates mean $\pm$ SE. Columns with identical letters are not significantly different ($P < 0.05$). (B) Effect of SA on biosynthesis of TFC in flax cell cultures. Values indicates mean $\pm$ SE. Columns with identical letters are not significantly different ($P < 0.05$)

FIGURE 4 Effect of SA on antioxidant potential of flax cell cultures. Values indicates mean $\pm$ SE. Columns with identical letters are not significantly different ($P < 0.05$)

highest antioxidant potential may be attributed to biosynthesis of these substrates in response to SA exposure. The mentioned polyphenols have been reported to break the oxidation chain reaction and quench free radicals [7,48]. Our study is

also in harmony with observations of [39], which reported that application of SA enhance antioxidant potential of *Catharanthus roseus*.

3.5 | Biosynthesis of lignans and neolignans

Lignans and neolignans are pharmacologically important polyphenols synthesized via phenylpropanoid pathway from a common precursor (coniferyl alcohol) [49]. Several elicitors have been employed to enhance the production of lignans and neolignans in cell cultures of *Linum* species [50–53]. Previously, flax cell cultures have also been exploited to stimulate the biosynthesis of Lignans and neolignans [20,29,33,51,54]. In the present study, in response to SA, flax cell cultures displayed considerable improvement in lignans and neolignans accumulation than their respective control. Biosynthesis of these metabolites was concentration-dependent and enhanced with SA exposure time. Maximum biosynthesis of SDG, LDG, DCG, and GGCG were noted when cell cultures were exposed to 50 μ M SA after 72 h treatment. When compared

FIGURE 5 (A) Effect of SA on SDG biosynthesis in cell cultures of flax. Columns with identical letters are not significantly different ($P < 0.05$). (B) Effect of SA on LDG biosynthesis in cell cultures of flax. Values indicates mean $\pm$ SE. Columns with identical letters are not significantly different ($P < 0.05$). (C) Effect of SA on DCG biosynthesis in cell cultures of flax. Values indicates mean $\pm$ SE. Columns with identical letters are not significantly different ($P < 0.05$). (D) Effect of SA on GGCG biosynthesis in cell cultures of flax. Values indicates mean $\pm$ SE. Columns with identical letters are not significantly different ($P < 0.05$)

to their controls, 2.7-fold increase in accumulation of SDG, 1.8-fold enhancement in LDG, and 3.98 DCG and 3.88 GGCG was noted in cell cultures of flax (Figure 5A–D). Our results are in harmony with previous reports, in which SA induced biosynthesis of lignans in cell cultures of *Linum album* [55]. They reported higher expression of phenylpropanoid vital enzymes PAL, CAD, and CCR mRNA which might be same in our case. In comparison antioxidant with previous studies of elicitation on flax lignans and neolignans [15,33,54], this is the highest amount SDG, DCG, and GGCG ever reported in cell cultures of flax.

4 | CONCLUDING REMARKS

To conclude, SA showed positive effects on biomass accumulation, TPC, and TFC. Moreover, our results also showed that moderate concentration of SA improved the biosynthesis of lignans and neolignans. Hence, a cost-efficient system for biosynthesis of pharmacologically attractive metabolites has been established.

ACKNOWLEDGMENTS

Dr. Bilal Haider Abbasi acknowledges research fellowship of Le Studium-Institute for Advanced Studies, Loire Valley, Orléans, France.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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How to cite this article: Nadeem M, Ahmad W, Zahir A, Hano C, Abbasi BH. Salicylic acid-enhanced biosynthesis of pharmacologically important lignans and neo lignans in cell suspension culture of *Linum usitatissimum* L. *Eng Life Sci* 2019;19:168–174. <https://doi.org/10.1002/elsc.201800095>