



## ***Trichoderma* strains- *Silybum marianum* hairy root cultures interactions**

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### **Abstract**

**Background and objectives:** Silymarin is a unique flavonoid complex with documented hepatoprotective properties. *Silybum marianum* hairy root culture as a source for producing silymarin has been an important strategy for study the cell signaling pathway. In the present investigation *Trichoderma* strains- *Silybum marianum* hairy root cultures interactions have been studied. **Methods:** The effects of two *Trichoderma* Strains (KHB and G46-7) (0, 0.5, 1, 2 and 4 mg/ 50 mL culture) in 6 different exposure times (0, 24, 48, 72, 96 and 120 h) have been investigated on flavonolignans production. The flavonolignans were analyzed by High Performance Liquid Chromatography method. Cell signaling pathway was evaluated by determination of H<sub>2</sub>O<sub>2</sub> content, peroxidase and ascorbate peroxidase activities. **Results:** The elicitation effects of two *Trichoderma* Strains (KHB and G46-7) were examined on flavonolignans accumulation and the activation of cell defense system in *S. marianum* hairy root cultures. The results indicated that the highest silymarin accumulation (0.45 and 0.33 mg/g DW) was obtained in media elicited with 0.5 mg/50 mL cultures of *T. harzianum* Strains (KHB and G46-3, respectively) after 120 h. Feeding time experiments indicated that a significant higher content of silymarin production was achieved after 120 and 72 h in media treated with 0.5 mg/50 mL cultures of KHB and G46-3, respectively. Our results showed that *S. marianum* treated by KHB strain, increased taxifolin, silychristin, isosilybin and silydianin productions significantly. The H<sub>2</sub>O<sub>2</sub> content in the control hairy root cultures remained lower than the treated cultures. There was significant enhancement in both peroxidase and ascorbate peroxidase activities in treated hairy roots reaching a peak after 72 h. **Conclusion:** These findings suggested that some *Trichoderma* strains are positive elicitors for promoting silymarin accumulation in *S. marianum* hairy root cultures. The results also suggested the presence of H<sub>2</sub>O<sub>2</sub> and oxidative burst induced by *T. harzianum* as a signaling pathway.

**Keywords:** elicitation, flavonolignans, signaling pathway, *Silybum marianum*

### **Introduction**

Milk thistle (*Silybum marianum* L. Gaertn), a plant of family Asteraceae, and native to the

Mediterranean and North African regions, is an annual herb and has been used medicinally as a

natural remedy for over 2,000 years [1]. A flavonoid complex called silymarin can be extracted from the seeds of milk thistle and is believed to be the biologically active component [2]. The flavonolignans are important hepatoprotective drugs widely used in human therapy of various liver damages [3]. Silymarin is also beneficial in reducing the chances for developing certain cancers [4].

Milk thistle tissue cultures could be an appropriate method for the production of flavonolignans [5]. Few efforts have been carried out to produce flavonolignans in tissue cultures of *S. marianum* [6-9]. In most cases silymarin production in *in vitro* cultures has been very low and has even disappeared in prolonged cultures. In all cases production has been lower than *S. marianum* dried fruits [5]. However, in some cases, elicitation of the culture medium has increased the production of the flavonolignans [10-12].

The major limitations of cell cultures are their instability during long-term culture [14]. Therefore many researchers have focused on transforming hairy root cultures by *Agrobacterium rhizogenes* [7,15]. The transformed roots have attractive properties for secondary metabolites production, compared to differentiated cell cultures [15,16]. Hairy roots are genetically stable and not repressed during the growth phase of the culture [17].

Enhancement of secondary metabolites production by elicitation is one of the recent strategies [13,18,19]. Several types of secondary metabolites have been elevated by elicitation, such as terpenoids [20], coumarin derivatives [21], alkaloids [22], and flavonoids [23-25]. As recently published [23,24,26] treatment of *S. marianum* hairy root cultures with different types of elicitors or feeding with precursors have improved production of silymarin. The mechanisms of elicitation are complex and there are many hypotheses regarding the modes of elicitors' actions. Previous studies have reported improvement of metabolite production by fungal elicitors in hairy root cultures [27,28].

Abiotic and biotic stresses activate the cell defense system and increase production of reactive oxygen species (ROS) [29]. ROS such as hydrogen peroxide ( $H_2O_2$ ), superoxide radical ( $O_2^-$ ) and hydroxyl radical ( $OH^-$ ) are toxic and cause damage to DNA, proteins, lipids, chlorophyll, etc [30]. Plant cells need to be protected from toxic effects of these ROS with antioxidant enzymes such as ascorbate peroxidase (APX), peroxidase (POD), superoxide dismutase (SOD), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and catalase (CAT) and non-enzymatic substances such as  $\alpha$ -tocopherol and ascorbic acid [30].

*Trichoderma* is a fungal genus, first described in 1794, that includes anamorphic fungi, isolated primarily from soil and decomposing organic matter [31]. Elicitors from *Trichoderma* activate the expression of genes involved in plant defense response system and promote the growth of the plant, root system and nutrient availability [32-34].

There is no report about the use of *Trichoderma* as an elicitor to increase silymarin accumulation in tissue cultures conditions. Little is known about silymarin production pathway and it is not clear what factors influence this signal transduction pathway. In order to investigate the possible plant cell-fungus extract interactions that may be effective on silymarin accumulation in *S. marianum* hairy root cultures, the effects of different strains and concentrations of *Trichoderma* were compared on *S. marianum* hairy root cultures.

## Experimental

### *Hairy root cultures*

Hairy root culture of *S. marianum* was transformed by *Agrobacterium rhizogenes* (AR15834), and the genetic transformation of these hairy roots was confirmed by polymerase chain reaction (PCR) according to the method described by Rahnema *et al.* [7]. Hairy roots cultures were induced by transferring six 1 cm roots to 50 mL of Murashige and Skoog liquid medium (MS) supplemented with 30 g/L sucrose in 150 mL flaks [35]. All experiments were

carried out on an orbital shaker set at 150 rpm and incubated at 25 °C in the dark.

#### *Fungal material*

*Trichoderma harzianum* isolates including KHB and G46-3, were used in this study. Isolate G46-3 was obtained from rice fields in Gilan (Rezvanshahr) and KHB from dead fallen leaves in Mazandaran (Polesefid). *Trichoderma* isolates were grown on potato dextrose agar medium (PDA) for 5 days in an incubator at 25 °C. Then the grown *Trichoderma* isolates were transferred to light conditions for 2 days. When colonies of the fungus were grown on plates, the medium with mycelium were cut into 50 mm plugs [36].

#### *Preparation and addition of elicitor*

The fungus elicitors were prepared according to Chong *et al.* [37]. Different content of each *Trichoderma* (0.5, 1, 2 and 4 mg) was dissolved in 50 mL MS medium. The solutions were sterilized by autoclaving at 120 °C and 1 atm over 20 min and used as elicitor. The elicitors were added to 30-days-old hairy root cultures. Controls received an equivalent volume of culture media. For a time course study, untreated and elicited hairy roots were harvested at different time intervals (0, 24, 48, 72, 96 and 120 h) and then frozen immediately at -80 °C for biochemical assay. Biomass was quantified by dry weight. Each experiment was repeated twice with 3 replicates each.

#### *Analytical procedures*

Silymarin was quantified by high performance liquid chromatography (HPLC). Analyses were carried out as described by Hasanloo *et al.* [6]. Standards of silymarin (SLM), silybin (SBN), iso silybin (ISBN) and taxifolin (TXF), were purchased from Sigma Aldrich; silycristin (SCN) and silydianin (SDN) from Phytolab.

#### *Determination of H<sub>2</sub>O<sub>2</sub> content*

Hydrogen peroxide content was determined according to Velikova *et al.* [38]. Frozen hairy roots were homogenized in an ice bath with 5 mL

of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12000 g for 15 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The absorbance of the supernatant was measured at 390 nm. The content of H<sub>2</sub>O<sub>2</sub> was calculated by comparison with a standard calibration curve previously plotted by using different concentrations of H<sub>2</sub>O<sub>2</sub>.

#### *Extraction and assay of enzymes*

The POD assay was carried out using the method of Chance and Maehly [39]. For the POD activity assay, enzyme extract was dissolved in 100 mM potassium phosphate buffer solution at pH 7.0, 10 mM Guaiacol and 70 mM H<sub>2</sub>O<sub>2</sub> solution at 100 mM potassium phosphate buffer (pH 7.0). The increase in absorbance was monitored at 420 nm. Total protein was assayed according to Bradford *et al.* [40] and the results were recorded base on  $\Delta$ OD/mg protein min.

APX activity was determined by estimating the rate of ascorbate oxidation as reported by Nakano and Asada [41]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM sodium ascorbate, 0.1 mM H<sub>2</sub>O<sub>2</sub> and the enzyme extract. Decrease in absorbance at 290 nm was measured at 25 °C for 3 min

#### *Statistical analysis*

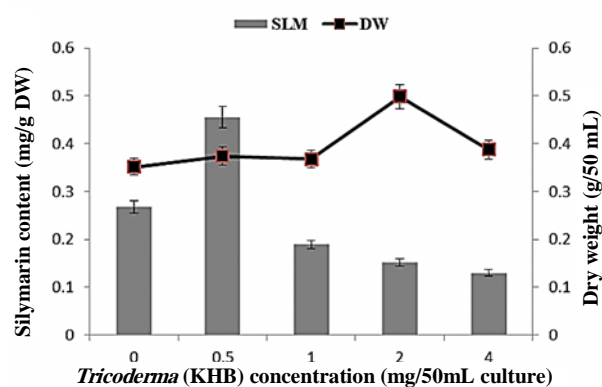
The data were given as the mean of at least three replicates. Statistical analysis was performed with SAS software (Version 6.2) using ANOVA method with Duncan test set at  $\alpha \leq 0.05$ .

## **Results and Discussion**

### *Effects of different concentrations of T. harzianum (KHB)*

Hairy root cultures (30 days old) were treated with four different concentrations (0, 0.5, 1, 2 and 4 mg/50 mL culture) of *T. harzianum* (KHB) for 120 h. Treatment of hairy roots with 2 mg/50 mL culture of *T. harzianum* (KHB) resulted in the highest amount of dry weight (0.498 g/50 mL) that was 1.41- fold greater than the corresponding controls. The maximum amount of silymarin accumulation (0.45 mg/g DW) was

obtained in hairy roots after 120 h in media supplemented with 0.5 mg/50 mL culture of *T. harzianum* (KHB) which was 1.7-fold greater than the control (0.267 mg/g DW) (figure 1). Also, the production of silymarin was lower than that of the control in media treated with 1, 2 and 4 mg/50 mL culture *T. harzianum* (KHB) (0.189, 0.152 and 0.130 mg/g DW, respectively). The content of ISBN, SBN, SCN, SDN and TXF in the samples treated with 0.5 mg/ 50 ml culture *T. harzianum* (KHB) were 0.033, 0.031, 0.052, 0.061, and 0.140 mg/g DW, respectively; while in non-treated hairy roots were 0.013, 0.013, 0.038, 0.031 and 0.041 mg/g DW, respectively (table 1). Based on the results obtained, the concentration of 0.5 mg/50 mL culture of *T. harzianum* (KHB) was chosen for further experiments.



**Figure 1.** Effect of different concentrations of *Tricoderma* (KHB) on silymarin accumulation and DW of *S. marianum* hairy root culture. Data are the average of three experiments, each performed in triplicate (means±SD).

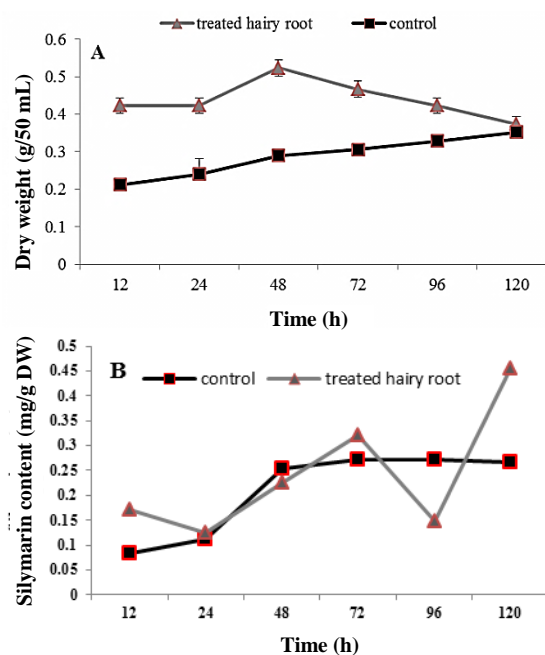
*Effects of T. harzianum (KHB) feeding time on growth index and silymarin production*

Time course for induction of silymarin and growth index in culture treated with 0.5 mg/50 mL culture medium of *T. harzianum* (KHB) have been presented in figure 2 (A and B).

*T. harzianum* (KHB) had a positive effect on the biomass, which was higher than the control (figure 2, A). The biomass production was enhanced after 24 h and reached a peak after 48 h which was 1.8-times higher than the control (0.289 g/50 mL). There was a gradual decline in

hairy root dry weight from 48 to 120 h in treated hairy roots. Also, the production of silymarin was higher than the control. *T. harzianum* (KHB) not only increased the biomass production but also induced the production of silymarin. A significant decrease was observed from 72 to 96 h and enhanced after 96 h and reached a peak after 120 h (0.455 mg/g DW) which was 1.7-fold higher than the control. Silymarin production in non- treated hairy roots showed no significant changes after 48 h (figure 2, B).

The highest content of TXF (0.123 and 0.188 mg/g DW, respectively) was obtained after 72 and 120 h in *T. harzianum* (KHB) treated media which was higher than the control (0.098 and 0.095 mg/g DW, respectively) (table 2). The highest SCN accumulation (0.084 mg/g DW) was observed in non-treated media after 72 h.



**Figure 2.** Time course of the *Tricoderma* (KHB) -induced biomass (A) and silymarin accumulation (B) of *S. marianum* treated and non-treated (control) hairy root cultures. Data are the average of three experiments; each in triplicate (means±SD).

SDN production increased upon stimulation and reached a maximum content after 72 and 120 h (0.071 and 0.070 mg/g DW, respectively). Our

**Table 1.** Flavonolignan content (mg/g DW) in *Trichoderma* (KHB)-treated and non-treated (control) hairy root cultures of *S. marianum*. Data show means±SD from triplicate experiments.

Trichoderma concentration	Flavonolignans				
	Taxifolin	Silychristin	Silydianin	Silybin	Isosilybin
0 (Control)	0.041± 0.05 <sup>d</sup>	0.038± 0.00 <sup>c</sup>	0.031± 0.00 <sup>c</sup>	0.013± 0.00 <sup>b</sup>	0.013± 0.01 <sup>c</sup>
0.5	0.140± 0.01 <sup>a</sup>	0.052± 0.01 <sup>a</sup>	0.061± 0.04 <sup>a</sup>	0.031± 0.01 <sup>a</sup>	0.033± 0.00 <sup>a</sup>
1	0.071± 0.08 <sup>b</sup>	0.044± 0.10 <sup>b</sup>	0.040± 0.02 <sup>b</sup>	0.019± 0.02 <sup>b</sup>	0.025± 0.03 <sup>b</sup>
2	0.052± 0.07 <sup>c</sup>	0.035± 0.00 <sup>c</sup>	0.021± 0.01 <sup>d</sup>	0.009± 0.02 <sup>c</sup>	0.012± 0.00 <sup>c</sup>
4	0.049± 0.00 <sup>d</sup>	0.028± 0.00 <sup>d</sup>	0.026± 0.01 <sup>d</sup>	0.008± 0.10 <sup>c</sup>	0.011± 0.02 <sup>cd</sup>

The superscript letters are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at  $p < 0.05$ .

**Table 2.** Flavonolignans content (mg/g DW) in *T. harzianum* (KHB) treated and non-treated (control) hairy root cultures of *S. marianum*. Data show means±SD from triplicate experiments.

Time (h)		Flavonolignans				
		Taxifolin	Silychristin	Silydianin	Silybin	Isosilybin
12	Treated	0.050±0.01 <sup>e</sup>	0.032± 0.001 <sup>de</sup>	0.041± 0.004 <sup>b</sup>	0.024± 0.004 <sup>d</sup>	0.022± 0.007 <sup>cd</sup>
	Control	0.017± 0.000 <sup>g</sup>	0.024± 0.00 <sup>c</sup>	0.022±0.002 <sup>d</sup>	0.009±0.00 <sup>e</sup>	0.010± 0.007 <sup>e</sup>
24	Treated	0.036±0.008 <sup>f</sup>	0.032±0.004 <sup>de</sup>	0.026±0.005 <sup>d</sup>	0.019±0.001 <sup>de</sup>	0.010±0.003 <sup>e</sup>
	Control	0.012± 0.002 <sup>g</sup>	0.024± 0.006 <sup>c</sup>	0.009±0.002 <sup>f</sup>	0.044± 0.006 <sup>c</sup>	0.020± 0.000 <sup>d</sup>
48	Treated	0.107±0.05 <sup>dc</sup>	0.056±0.01 <sup>a</sup>	0.049±0.01 <sup>b</sup>	0.024±0.01 <sup>d</sup>	0.026±0.002 <sup>cd</sup>
	Control	0.088± 0.006 <sup>de</sup>	0.044± 0.006 <sup>d</sup>	0.021±0.002 <sup>d</sup>	0.070± 0.008 <sup>a</sup>	0.028± 0.002 <sup>cd</sup>
72	Treated	0.123± 0.03 <sup>b</sup>	0.053±0.004 <sup>cd</sup>	0.071± 0.004 <sup>a</sup>	0.036± 0.001 <sup>c</sup>	0.034± 0.005 <sup>c</sup>
	Control	0.098± 0.009 <sup>d</sup>	0.084± 0.007 <sup>a</sup>	0.025±0.007 <sup>d</sup>	0.046± 0.008 <sup>c</sup>	0.040± 0.02 <sup>b</sup>
96	Treated	0.052±0.01 <sup>e</sup>	0.035± 0.008 <sup>de</sup>	0.030± 0.008 <sup>c</sup>	0.013± 0.001 <sup>de</sup>	0.016± 0.004 <sup>e</sup>
	Control	0.098± 0.009 <sup>d</sup>	0.039± 0.001 <sup>d</sup>	0.016±0.005 <sup>e</sup>	0.069± 0.002 <sup>b</sup>	0.046± 0.006 <sup>a</sup>
120	Treated	0.188± 0.06 <sup>a</sup>	0.060± 0.01 <sup>c</sup>	0.070± 0.01 <sup>a</sup>	0.038± 0.01 <sup>c</sup>	0.044± 0.01 <sup>ab</sup>
	Control	0.095±0.005 <sup>d</sup>	0.070± 0.00 <sup>b</sup>	0.030± 0.00 <sup>c</sup>	0.040± 0.0068 <sup>c</sup>	0.029± 0.007 <sup>cd</sup>

The superscript letters are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at  $p < 0.05$ .

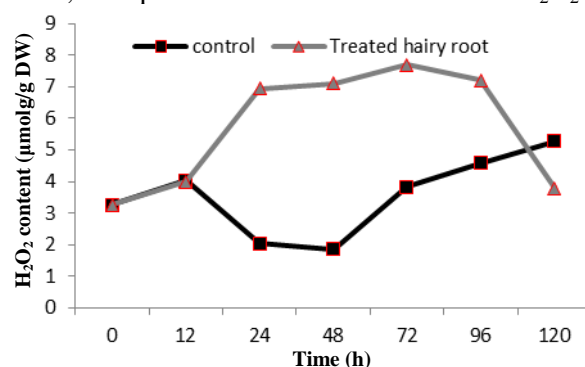
results showed that silybin production in treated hairy root cultures was lower than the non-treated root cultures after 24 h. ISCN production was enhanced after 120 h (0.044 mg/g DW) that was 2.9 times higher than the non- treated hairy root cultures (table 2).

*H<sub>2</sub>O<sub>2</sub> content, peroxidase and ascorbate peroxidase activity in T. harzianum (KHB) treated hairy roots*

To determine how *T. harzianum* (KHB) stimulates silymarin accumulation and how the ROS signaling pathway is also an integral part of the elicitor signaling pathway leading to the accumulation of silymarin, H<sub>2</sub>O<sub>2</sub> content, the activity of peroxidase and ascorbate peroxidase activity were assayed.

Figure 3 indicates the H<sub>2</sub>O<sub>2</sub> content in *T. harzianum* (KHB) treated and non- treated hairy

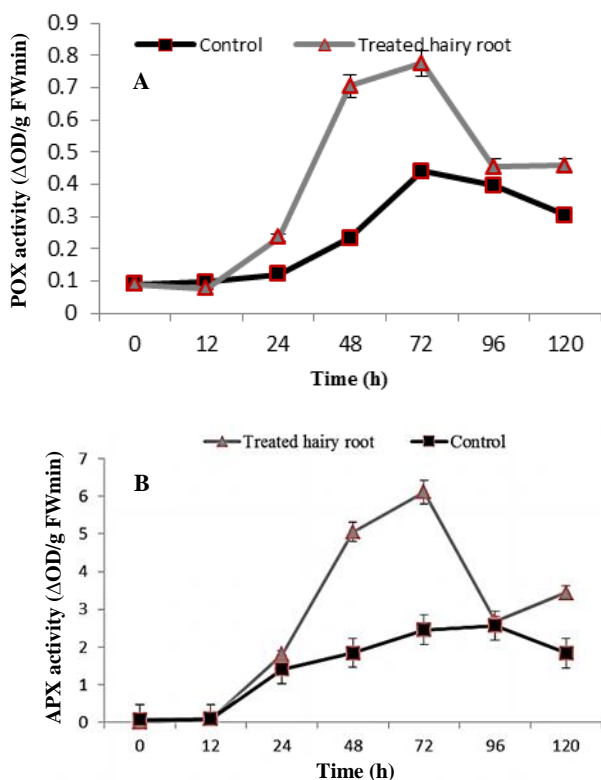
roots within the period of 120 h. As an overall trend, it is quite obvious that the content of H<sub>2</sub>O<sub>2</sub>



**Figure 3.** Time-course of H<sub>2</sub>O<sub>2</sub> variations in *S. marianum* hairy root cultures treated with *Trichoderma* (KHB). Data show means±SD from triplicate experiments.

dramatically rose, reaching a peak (6.95 µM/g DW) after 24 h that was 3-times that of the

control (2.03  $\mu\text{M/g DW}$ ). There was a gradual decline in  $\text{H}_2\text{O}_2$  content from 96 h to 120 h in treated hairy roots. The  $\text{H}_2\text{O}_2$  content in non-treated hairy roots was changed after 12 h and declined from 12 to 24 h. There was no significant change in  $\text{H}_2\text{O}_2$  content between 24 and 48 h, however, after 48 h  $\text{H}_2\text{O}_2$  content gradually increased. The changes of  $\text{H}_2\text{O}_2$  content under *T. harzianum* (KHB) treatment were significantly higher than the non- treated hairy roots. As shown in figure 4A, the peroxidase activity was activated by *T. harzianum* (KHB) and reached to an extremely high level (0.775  $\Delta \text{OD/g FWmin}$ ) after 72 h of treatment that was 1.76- fold higher than the control (0.439  $\Delta \text{OD/g FW min}$ ) then decreased.



**Figure 4.** Time-course of Peroxidase (A) and ascorbate peroxidase (B) activity in *S. marianum* hairy root cultures treated with Tricoderma (KHB). Data show means  $\pm$  SD from triplicate experiments.

The changes of  $\text{H}_2\text{O}_2$  content in non- treated hairy roots were similar to treatment experiments. However, addition of elicitor increased  $\text{H}_2\text{O}_2$

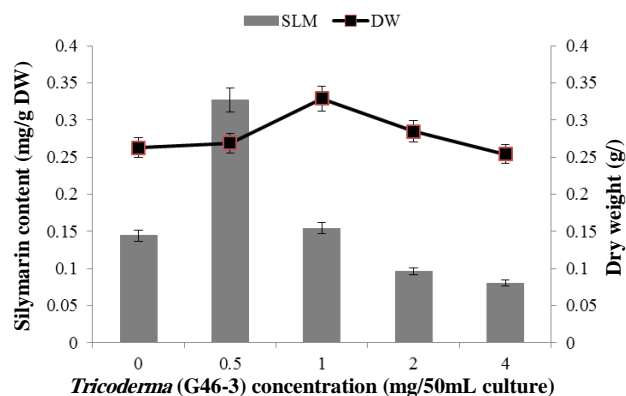
activity after 12 h treatment significantly. Figure 4B indicates the ascorbate peroxidase activity in treated and non-treated hairy roots within the period of 120 h. As shown in figure 4B the treated hairy roots showed significantly more ascorbate peroxidase activity after 24 h than the non-treated hairy roots, hitting a peak (6.11  $\Delta \text{OD/g FW min}$ ) after 72 h that was 2.48-times that of the control (2.46  $\Delta \text{OD/g FW min}$ ). There was a gradual decline in ascorbate peroxidase activity from 72 h to 96 h in treated hairy roots (2.68  $\Delta \text{OD/g FW min}$ ). The ascorbate peroxidase activity in non-treated hairy roots reached a peak after 96 h (2.57  $\Delta \text{OD/g FW min}$ ) but declined from 96 h to 120 h dropping to 1.84  $\Delta \text{OD/g FW min}$ .

*Effects of different concentrations of T. harzianum (G46-3)*

The results obtained from the hairy root cultures (30 days old) after 120 h treatment with five different concentrations (0.5, 1, 2 and 4 mg/50 mL culture) of *T. harzianum* (G46-3) are presented in figure 5. There were no significant differences between DW of *T. harzianum* (G46-3) treated hairy root (0.5 mg/50 mL culture) and non- treated hairy roots (0.262 and 0.268 g/50 mL, respectively). An increase in DW (0.328 g/50 mL) was observed by the addition of 1 mg/50 mL culture *T. harzianum* (G46-3). Negative correlation was found between DW and high concentration of *T. harzianum* (G46-3) (2 and 4 mg/50 mL culture). The DW decreased to 0.253 g after 120 h supplemented with 4 mg/50 mL culture *T. harzianum* (G46-3).

Figure 5 compares silymarin content in *T. harzianum* (G46-3) treated and non- treated hairy roots. The most striking result to emerge from figure 5 is that, the highest silymarin accumulation (0.326 mg/g DW) was obtained in media elicited with 0.5 mg / 50 ml culture *T. harzianum* (G46-3). The overall response to higher concentration (1, 2 and 4 mg/50 mL culture) of *T. harzianum* (G46-3) was negative and silymarin production was significantly decreased from 0.328 in media treated with 0.5

mg/50 mL culture *T. harzianum* (G46-3) to 0.080 mg/g DW in media elicited with 4 mg / 50 mL culture. As can be seen from the table 3, the content of SBN, ISBN, SCN, SDN and TXF in



**Figure 5.** Effect of different concentrations of *Trichoderma* (G46-3) on silymarin accumulation and DW of *S. marianum* hairy root culture. Data are the average of three experiments, each performed in triplicate (means  $\pm$  SD).

the samples treated with 0.5 mg/50 mL culture *T. harzianum* (G46) were 0.025, 0.031, 0.034, 0.035 and 0.084 mg/g DW, respectively while in non-treated hairy roots were 0.015, 0.009, 0.038, 0.034 and 0.044 mg/g DW, respectively. Based on the results obtained, the concentration of 0.5 mg/50 mL culture of *T. harzianum* (G46-3) was chosen for further experiments.

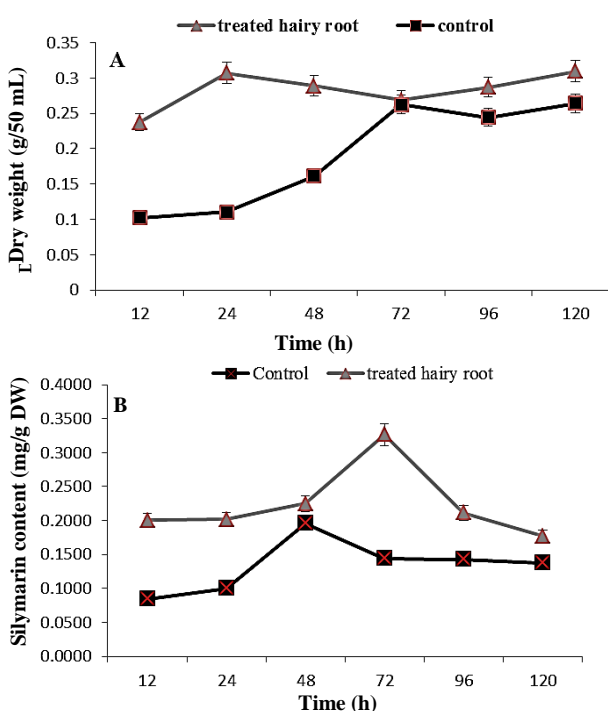
*Effects of T. harzianum (G46-3) feeding time on growth index and silymarin production*

Time course for induction of silymarin and biomass production in cultures treated with 0.5 mg/50 mL culture *T. harzianum* (G46-3) are presented in figure 6 (A and B). The elicitor had a positive effect on the DW (0.237 g/50 mL) after 12 h, which was higher than the control (0.102 g/50 mL). The DW was slightly decreased after 24 h and reached to a minimum DW after 72 h that had the same content as in the control. However, the DW content was enhanced after 72 h.

The content of silymarin in treated cultures was higher than the control from the beginning to the end of our experiment (figure 6B). The silymarin content showed an increase after 48 h and a

significant higher content of silymarin (0.326 mg/g DW) was achieved after 72 h. A reduction in silymarin content was observed after 72 h but remained higher than those of the non- treated cultures.

TXF, SCN, SDN and ISB production increased upon stimulation and reached a maximum content after 72 h (0.107, 0.054, 0.090 and 0.030 mg/g DW, respectively) (table 4). Our results showed that SBN production was elicited after 12 h (0.080 mg/g DW) (table 4).



**Figure 6.** Time course of the *Trichoderma* (G46-3) -induced biomass (A) and silymarin accumulation (B) of *S. marianum* treated and non-treated (control) hairy root culture. Data are the average of three experiments each in triplicate (means $\pm$ SD).

*H<sub>2</sub>O<sub>2</sub> content, ascorbate peroxidase and peroxidase activity in T. harzianum (G46- 3) treated hairy roots*

As shown in figure 7, H<sub>2</sub>O<sub>2</sub> content increased upon stimulation by *T. harzianum* (G46-3). The H<sub>2</sub>O<sub>2</sub> content reached a pick after 72 h (7.311  $\mu$ M/g DW) that was 1.91- fold greater than the corresponding control. The H<sub>2</sub>O<sub>2</sub> content in the

**Table 3.** Flavonolignan content (mg/g DW) in *Tricoderma* (G46-3)-treated and non-treated (Control) hairy root cultures of *S. marianum*. Data show means±SD from triplicate experiments.

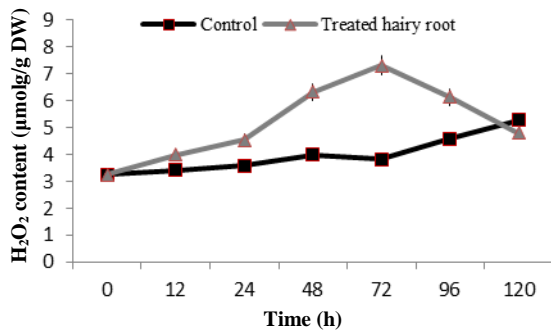
Tricoderma concentration	Flavonolignans				
	Taxifolin	Silychristin	Silydianin	Silybin	Isosilybin
0 (Control)	0.044± 0.05 <sup>c</sup>	0.038± 0.00 <sup>ab</sup>	0.034± 0.02 <sup>a</sup>	0.015± 0.00 <sup>b</sup>	0.009± 0.00 <sup>b</sup>
0.5	0.084± 0.01 <sup>a</sup>	0.034± 0.01 <sup>a</sup>	0.035± 0.04 <sup>a</sup>	0.025± 0.01 <sup>a</sup>	0.031± 0.00 <sup>a</sup>
1	0.072± 0.08 <sup>b</sup>	0.028± 0.10 <sup>c</sup>	0.035± 0.02 <sup>a</sup>	0.019± 0.02 <sup>b</sup>	0.025± 0.03 <sup>b</sup>
2	0.026± 0.00 <sup>d</sup>	0.023± 0.00 <sup>cd</sup>	0.022± 0.01 <sup>b</sup>	0.010± 0.01 <sup>c</sup>	0.007± 0.00 <sup>bc</sup>
4	0.027± 0.00 <sup>d</sup>	0.023± 0.00 <sup>cd</sup>	0.025± 0.01 <sup>b</sup>	0.010± 0.01 <sup>c</sup>	0.009± 0.02 <sup>b</sup>

The superscript letters are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at  $p < 0.05$ .

**Table 4.** Flavonolignans content (mg/gDW) in *T. harzianum* (G46-3) -treated and non-treated (Control) hairy root cultures of *S. marianum*. Data show means ± SD from triplicate experiments.

Time (h)		Flavonolignans				
		Taxifolin	Silychristin	Silydianin	Silybin	Isosilybin
12	Treated	0.017±0.001 <sup>gh</sup>	0.047±0.002 <sup>b</sup>	0.034±0.003 <sup>d</sup>	0.080± 0.010 <sup>a</sup>	0.020±0.000 <sup>ab</sup>
	Control	0.023± 0.002 <sup>g</sup>	0.020± 0.003 <sup>d</sup>	0.014± 0.003 <sup>f</sup>	0.016± 0.002 <sup>d</sup>	0.008± 0.004 <sup>d</sup>
24	Treated	0.084±0.003 <sup>b</sup>	0.036±0.001 <sup>c</sup>	0.052±0.001 <sup>c</sup>	0.014±0.003 <sup>d</sup>	0.014±0.002 <sup>c</sup>
	Control	0.024± 0.001 <sup>g</sup>	0.029± 0.014 <sup>cd</sup>	0.022± 0.008 <sup>e</sup>	0.014± 0.003 <sup>d</sup>	0.009± 0.02 <sup>d</sup>
48	Treated	0.056±0.003 <sup>d</sup>	0.045±0.002 <sup>b</sup>	0.065±0.003 <sup>b</sup>	0.041±0.005 <sup>b</sup>	0.015±0.006 <sup>c</sup>
	Control	0.045± 0.002 <sup>e</sup>	0.042± 0.005 <sup>b</sup>	0.062± 0.010 <sup>b</sup>	0.026± 0.020 <sup>c</sup>	0.020± 0.004 <sup>b</sup>
72	Treated	0.107±0.008 <sup>a</sup>	0.054±0.002 <sup>a</sup>	0.090±0.004 <sup>a</sup>	0.043±0.002 <sup>b</sup>	0.030±0.007 <sup>a</sup>
	Control	0.065± 0.010 <sup>c</sup>	0.030± 0.002 <sup>c</sup>	0.020± 0.004 <sup>e</sup>	0.018± 0.001 <sup>d</sup>	0.009± 0.020 <sup>d</sup>
96	Treated	0.080±0.005 <sup>b</sup>	0.033±0.004 <sup>c</sup>	0.037±0.006 <sup>d</sup>	0.027±0.004 <sup>c</sup>	0.025±0.014 <sup>b</sup>
	Control	0.076± 0.012 <sup>bc</sup>	0.025± 0.001 <sup>b</sup>	0.019± 0.010 <sup>ef</sup>	0.014± 0.003 <sup>d</sup>	0.008± 0.001 <sup>d</sup>
120	Treated	0.079±0.009 <sup>bc</sup>	0.029±0.006 <sup>cd</sup>	0.036±0.006 <sup>d</sup>	0.019±0.003 <sup>d</sup>	0.011±0.001 <sup>c</sup>
	Control	0.038± 0.002 <sup>f</sup>	0.042± 0.001 <sup>b</sup>	0.020± 0.030 <sup>c</sup>	0.021± 0.013 <sup>c</sup>	0.014± 0.005 <sup>c</sup>

The superscript letters are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at  $p < 0.05$ .



**Figure 7.** Time-course of H<sub>2</sub>O<sub>2</sub> variations in *S. marianum* hairy root cultures treated with *Tricoderma* (G46-3). Data show means±SD from triplicate experiments.

control remained lower than the treated cultures without marked changes. The peroxidase activity was increased by *T. harzianum* (KHB) and reached to an extremely high level (2.896 Δ

OD/g FW min) after 12 h of treatment that was 29.85- fold higher than the control (0.097 Δ OD/g FW min) and then gradually decreased from 12 to 24 h (figure 8A).

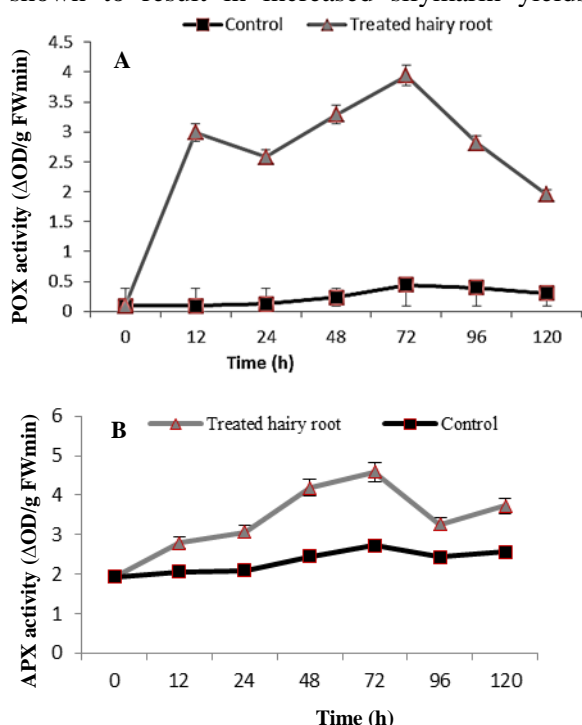
Total peroxidase activity was increased from 24 to 72 h, and then towards the end of the period it dropped sharply. There was a slight increase in the amount of the peroxidase activity in non-treated cultures after 48 h but became stable after that.

Figure 8B represents the ascorbate peroxidase activity in treated and non-treated hairy roots within the period of 120 h. As it can be seen from figure 8B, the treated hairy roots showed significantly more ascorbate peroxidase activity than the non-treated hairy roots, reaching a peak (4.581 Δ OD/g FW min) after 72h that was 1.67-times that of the control (2.731 Δ OD/g FW min). There was a gradual decline in ascorbate



peroxidase activity from 72 h to 96 h in treated hairy roots (3.262  $\Delta$  OD/g FW min) but then the trend was upward.

There was a slight increase in the ascorbate peroxidase activity in non-treated hairy roots reaching a peak at 72 h (2.731  $\Delta$  OD/g FW min). Elicitation of differentiated cell cultures may open new ways for the improvement of secondary metabolites. Hairy root cultures of *S. marianum* represent a valuable source for production of flavonolignans. All other elicitation studies on hairy root cultures were previously shown to result in increased silymarin yields.



**Figure 8.** Time-course of peroxidase (A) and ascorbate peroxidase (B) activity in *S. marianum* hairy root cultures treated with *Trichoderma* (G46-3). Data show means $\pm$ SD from triplicate experiment.

The greatest increases in silymarin accumulation were observed in the presence of salicylic acid (6 mg SA/50 mL culture) [23]. Our experiments with *Trichoderma* as a fungal elicitor used in hairy root culture of *S. marianum* showed changes in flavonolignan complex production. Treatment of hairy root cultures with 0.5 mg/50

mL culture *T. harzianum* (KHB) has improved production of silymarin to about 1.7-fold higher than that of the control. Maximum content of silymarin was 0.326 mg/g DW after 96 h in media treated with *T. harzianum* (G46-3).

The results of this investigation illustrate the signaling pathway acting as an integral signal and elicitor signal transducer for silymarin production. The current study suggests the presence of H<sub>2</sub>O<sub>2</sub> and oxidative burst induced by *T. harzianum*. The oxidative burst, during which large quantities of reactive oxygen species (ROS) like superoxide, hydrogen peroxide, hydroxyl radicals, peroxy radicals, alkoxy radicals, singlet oxygen, etc. are generated, is one of the earliest responses of plant cells under various abiotic and biotic stresses and natural course of senescence [42]. Wu and Ge have suggested that oxidative burst is an upstream event to Jasmonic acid (JA) accumulation, and both ROS from the oxidative burst and JA from the LOX pathway are key signal elements in the elicitation of taxol production of *Taxus chinensis* cells by low-energy ultrasound [43]. Goâmez-Vaâsquez *et al.* suggested that the production of ROS such as H<sub>2</sub>O<sub>2</sub> is responsible for elicitation process [44]. This report has been supported by Low and Merida who observed that involvement of ROS in cross linking of cell wall bound protein rich components can act as a secondary messenger and it is involved in activation of defense genes [45].

ROS communicate with other growth factors and the pathway forming part of the signaling network that controls responses downstream of ROS, ultimately influence growth and development. The dry weight of *S. marianum* root cultures treated with *T. harzianum* showed negative correlation with feeding time (after 48 h). Such results have already been reported in *Phytophthora cinnamomi* elicited *Hypericum perforatum* cell suspension cultures [46]. It has been reported that the reduction in biomass might be due to production of ROS [47]. In our study, H<sub>2</sub>O<sub>2</sub> content, POX and APX activity were increased. The plant cells are usually protected

against the effects of oxygen species using scavenging system. In addition, SLM content was found to be higher in elicited cultures than the control. All these data suggest that, exogenous treatment of *S. marianum* root cultures with *T. harzianum* strains stimulated the enzymatic and non-enzymatic system in *S. marianum* cultures.

According to this study, maximum activity of POX was observed in 72 h. The activity level of POX in the control remained lower than those of the treated cultures. APX activity dramatically increased after 24 h in treated hairy root cultures of *S. marianum*. It can thus be suggested that production of ROS is through the action of NADH dependent POD [48]. However, further research should be performed and this is an important issue for future researches.

Navazio *et al.* have indicated that secreted fungal molecules are sensed by plant cells through intracellular  $Ca^{2+}$  changes [49]. The specificity of the changes that they have recorded in the single and two-fungal elicitors indicated that this intracellular messenger delivers different messages to cells. A specificity of the perception mechanism by plant cells is confirmed by the fact that different patterns of intracellular ROS accumulation and cell death induction were stimulated by the various fungal elicitors. Garcia-brugger *et al.* have indicated that different intracellular events do not necessarily imply that the cascade of events follow independent pathways and an overlapping pathway might be activated [50].

The molecular nature of the elicitors produced by *Trichoderma* strains has been at least partially unraveled. The various components (polysaccharide, protein, lipid and ions) isolated from the cultured fungal mycelia have been studied on the plant cell growth and metabolites production [51]. Some of these compounds have been tested for their ability to induce expression of plant defense genes and disease resistance [52]. The 3 KDa *Trichoderma* fraction including trichorzianines A<sub>1</sub> and B<sub>1</sub>, have shown to be affecting elements on membrane permeability

and cell death which may result in cytoplasmic leakage through these ion channels [27]. Ming *et al.*, indicated that both extract of mycelium and the polysaccharide fraction promoted hairy root growth and stimulated the biosynthesis of tanshinones in hairy root cultures of *Salvia miltiorrhiza*. It was reported that PSF is one of the main active constituents responsible for promoting hairy root growth, as well as stimulating biosynthesis of tanshinones in the hairy root cultures [17].

In conclusion, elicitation of a medium with *Trichoderma* offers the possibility to enhance the content of some components of silymarin complex and flavonoid-taxifolin in *S. marianum* cultures *in vitro*. Both the type and concentration of fungal elicitors are very important in determining the enhancement of silymarin accumulation in the *S. marianum* hairy root culture. The application of fungal elicitors to *S. marianum* hairy root culture could be a useful tool for studying the regulation of flavonolignan production pathway and identification of key steps participating in the signaling network activated by the elicitor.

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#### Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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