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Research Article

Optimization of *Silybum marianum* L. callus production and magnifying callus silymarin accumulation by elicitors or precursors

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ABSTRACT

In the present work plant tissue culture is used as a powerful technology to synthesize pharmacologically relevant secondary compounds from *Silybum marianum* L. This plant produces silymarin, highly effective hepato-protective compound. Tissue culture medium composition was modified to explore the possibility to increase biomass production of *S. marianum callus* and eventually to enhance the accumulation and yield of silymarin. In a systematic approach, levels of asparagine(Asn), 6-benzyl aminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D) and myo-inositol were altered and callus growth was assessed together with levels of nitrogen metabolites especially total soluble amino acids, protein and flavolignan silymarin. Supplementation with 2,4-D dominated high callus growth characteristics. 2,4-D could partly be substituted by other growth regulators. The best medium (F) which produced the greatest callus growth consisted of 0.25 mg/l 2,4-D with 50 mg/l Asn, 0.05 mg/l BAP and 50 mg/l inositol. Additional improvement of the medium was achieved through addition of elicitors and precursors to (F media) which also enhanced the silymarin accumulation. Phenylalanine at 25 µM as precursor increased silymarin accumulation by 31% while the elicitation with glutathione at 0.1mM after 8 days and ascorbate at 0.5 mM after 8 days increased silymarin accumulation by 6.4 and 30.6 % respectively. Thus medium F in combination with 25 µM phenylalanine or 0.5 mM ascorbate in short term experiments led to optimal callus mass production with reasonable high content of silymarin.

Key words: Callus induction, Elicitors, Phenylalanine, Glutathione, Ascorbate, 2,4-D, Silymarin, 6-Benzylaminopurine, Asparagine.

INTRODUCTION

Viral liver diseases pose a serious health problem in many countries, in extreme cases causing cirrhosis and liver cancer. Clinical studies showed that 70 to 90% of patients with chronichepatitis and cirrhosis had hepatitis C virus infections which are considered to represent the dominant health problem in Egypt[1]. Thus there is a demand for liver medicines which however often are very expensive and poor people can hardly afford them. Thus, it is important to explore and exploit alternative sources for pharmaceutical products using natural resources and technologies. *Silybum marianum* is commonly known as milk thistle and belongs to the Asteraceae. *S. marianum* is one of the most important and best researched plants of ancient times and is used for the treatment of liver and gallbladder disorders, including hepatitis, cirrhosis and jaundice[2].*Milk Thistle Plus* produced from *S. marianum* effectively supports liver cell regeneration, enhances the liver's detoxification ability, restores liver functions, and strengthens the

immune system [3]. It contains the optimal combination of ingredients for the detoxification and protection of the liver. The main active compounds of milk thistle are silymarin and phenolic acids [3-4].Silymarin consists of a mixture of flavonolignan compounds, the most abundant constituents are silibinin, isosilibinin, silicristin and silidianin[5].

S. marianum geno types are able to grow in diverse habitats suggesting that ecotypes with more favorable amounts of flavolignan may be found expressing preferred traits for domestication and pharmacological purposes. However despite the economical and pharmaceutical value of *S. marianum* successful efforts for domestication and breeding of this species are scarce[6].The efficiency of seed germination and the rate of seedling growth are low and poorly controllable in *S. marianum*. Both growth parameters highly depend on various biological and environmental factors [7-8]. Field-grown plants are affected by seasonal and somatic variations as well as environmental pollutants that may alter their medicinal value.

Plant tissue culture is a powerful tool for addressing basic questions and solving applied problems in plant biotechnology. The *in vitro* production of plant secondary metabolites is achieved under controlled conditions and thus does not dependent on environmental fluctuations[9-10]. During the last decades, micro-propagation and other *in vitro* methods became popular in commercial horticulture and agriculture for mass propagation of certain crop plants [11-12]. Plant cell culture offers advantages over traditional field cultivation and chemical synthesis, particularly due to the facts that many natural compounds are either derived from slow-growing plants or from plants requiring elaborated growth conditions or because the active compounds are difficult to be synthesized with chemical methods[13-14]. All these criteria apply to *S. marianum*, so that an *in vitro* culture system producing pharmacologically relevant compounds could be of major interest.

Major limitations of *S. marianum* cell cultures come from their instability during long-term culture and low product yield[10]. In order to increase yields for commercial exploitation, efforts have focused on the stimulation of biosynthetic activities of cultured cells using various methods [15-16]. Modified chemical and physical factors concerned the media components, phytohormones supplementations, pH, temperature and aeration and their effects on production of secondary metabolites have been extensively studied[17-20]. Optimization is particularly useful when the precursors are expensive. In this respect, several products were found to accumulate in cultured cells to higher level than in native plants through optimization of cultural conditions.

In the present work, we analyzed the production of the liver protective compound silymarin in tissue cultures of Egyptian *S. marianum* (L.) with the main aim to establish a protocol for high yield production. To this end accumulation of this medical constituent was assessed in cultured tissues of *S. marianum* in dependence on growth time, medium composition and medium supply in combination with the effectors as phenylalanine, glutathione and ascorbate.

MATERIALS AND METHODS

Seed collection and preparation of explants: Seeds of *S. marianum* were collected from naturally growing plants in Gharbiya district Egypt during the growing season 2011/12. Seeds were washed several times with distilled water and imbibed in distilled water for 1 h at 37°C to ease the removal of the testa without injuring the cotyledons. Following testa removal the two cotyledons were separated from each other. All steps took place in a laminar flow chamber. Cotyledons were surface-sterilized by three successive washes with ethanol (80% v/v) followed by Clorox (5% v/v) for 50 several wash steps in sterilized distilled water. Subsequently, sterilized cotyledons were cultured on solidified Murashige and Skoog (MS) basal medium.

Preparation of media: Murashige and Skoog (MS) basal medium (4.4 g/l) was supplemented with 30 g/l carbon source (sucrose) and four concentrations of each 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 0.25, 0.5, 1.0 mg/l), 6-benzylaminopurine (BAP)(0, 0.01, 0.05, 0.1 mg/l), asparagine (0, 25, 50, 100 mg/l) and myo-inositol (0, 25, 50, 100 mg/l) for obtaining 16 (4*4) different media. The medium free of all growth regulators and medium with absence of 2,4-D in the presence of low concentrations of Asn, BAP and inositol were excluded since callus initiation was unsuccessful. The different concentrations of 2,4-D and BAP were obtained by diluting the stock solutions prepared at 0.5 and 0.05 mg/ml in ethanol (85%) respectively. Asparagine dissolved in drops of 1 M HClwas prepared as 50 mg/ml stock solution, myo-inositol at 50 mg/ml in distilled water. Each medium was labeled with alphabetical letters from A through O.

The pH of the medium was adjusted to 5.8 (using NaOH or HCl). The media were solidified by supplementation with 7 g/l agar. All media were autoclaved at 121℃ and a pressure of 1.2 kg cm^{-2} for 20 min. Media (40 ml) were poured in sterilized conical 100 ml flasks before complete solidification.

The previously sterilized cotyledons were then cultured on solidified (MS) medium. Each treatment was replicated nine times. All cultures were incubated in the growth chamber at 25±2°C in darkness for 2 months during which callus was transferred to new medium after 21, 42 and 63 days of culturing. At these time points three flasks of each treatment were harvested for callus measurements.

Precursor and elicitors: Precursor and two elicitors, namely as phenylalanine, glutathione and ascorbate were added to the medium which led to high callus production (media F supplemented with growth regulators of 0.25 mg/l 2,4-D, 50 mg/l asparagine, 0.05 mg/l BAP and 50 mg/l myo-Inositol).

Phenylalanine precursor: A stock solution of 10 mM concentration was prepared by dissolving phenylalanine in distilled water, sterilized using 0.2µm micro filters and concentrations of 0, 1, 10, 25 and 100 µM prepared.

Glutathione & ascorbate: Glutathione and ascorbate stock solutions of 1 mg/ml (w/v) concentrations were prepared by dissolving 10 mg of glutathione and ascorbatein 10 ml of distilled water, filter sterilized using 0.2µm micro filters and used for preparing media with final concentrations 0, 0.1, 0.5, 1.0 and 2.0 mM. Glutathione and ascorbate were added to media F with long and short term designed experiments. In long term, explants of *S. marianum* cotyledons were cultured on solidified MS media (F medium) supplemented with different concentrations of glutathione and ascorbate. While in short term experiments glutathione and ascorbate were applied to cultured callus with age 42 day on media F. Then callus was harvested 4, 8, 12 and 16 days after the treatments.

Growth parameters: Characteristics of induced calli in all treatments were measured at 21 day intervals for two months. Measured parameters were callus fresh weight (FW),dry weight (DW), water content (WC), growth rate (GR), percentage of callus induction (PCI), day of callus induction (DCI), mortality after first cutting (MFC), mortality after second cutting (MSC), growth rate after first cutting (GRFC) and growth rate after second cutting (GRSC). At harvest time the calli of the three flasks from each treatment were freed from medium, and washed with distilled water and used for the determination of callus fresh weight. Afterwards calli were dried at 40°C until constant weight was reached and their weights were recorded.

Relative water content (%RWC): Callus samples of known fresh weight were dried in an oven set at 70°C for 24 h and RWC was calculated by the following formula:

$$\%RWC = 100^{*}(FW\text{-}DW)/DW$$

Callus growth rate (%GR): was calculated according to equation

$$\%GR = 100^{*}[(FW_F\text{-}FW_I)/FW_I]/(T_F\text{-}T_I)$$

Where I and F were the initial and final callus weight and T is the time.

Percentage of callus induction (PCI): was evaluated after induction of explants in Petri dishes [21]. Percentage of callus induction was calculated with the following equation:

%PCI = 100^{*}(number of cotyledons producing callus/number of cultured cotyledons planted in Petri dish).

Day of callus inductions (DCI) was obtained by monitoring appearance of the first cell amplifications that could be detected with naked eye. Day callus induction = Number of days from culturing until callus induction.

Regeneration ability of callus was determined through cutting callus into two pieces each 21st day and re-cultivation on new medium. The percentage of growth rate after first and second cutting were measured as previously mentioned. Callus mortality after first and second cutting was recorded.

Callus volumes (V cm^3): were measured by using measuring cylinder containing specific volume of water and the increase in water level in the cylinder was recorded and represented the callus volume per cm^3.

Callus bulk density: was calculated according to equation

(CBD mg/cm^3) = Callus dry weight (mg)/callus volume (cm^3)

Spectrophotometric measurement of Silymarin: The content of silymarin compounds in *S. marianum* callus was tentatively quantified[22]. The methanol extract was evaporated under a hood. The dried residue was redissolved in 1 ml methanol. Then 0.5 ml of 2,4-dinitrophenylhydrazine-sulfuric acid (DNPH) reagent was pipetted to 0.25 ml of the methanolic solution and placed in a water bath of 55°C for 50 min. After cooling to room temperature 1.75 ml of KOH-methanol (10%v/v) was added. Subsequently, the complete assay solution was transferred to a centrifuge tube. Following addition of 5 ml methanol, the mixture was centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was measured at 490 nm with a spectrophotometer (Cary 300, version 9, Agilent, Santa Clara, CA, USA).

The silymarin content (calculated as silibinin) was calculated by the following formula:

% silymarin = A × 25/($\varepsilon\frac{1\%}{1\,cm}$ × DW) = (A*10*7.5*10^{-3})/ (585*G)

where A is the absorption at 490 nm, 585 is the specific absorption coefficient and G is the mass of the sample (g).

Quantitative estimation of total soluble proteins: The total soluble proteins content was estimated quantitatively in borate buffer extract. Using 0.1 ml (borate buffer extract) was mixed with 3 ml Coumassie Brilliant Blue reagent[23]. The absorbance was measured spectrophotometrically at 595nm after 2 min. The protein content was calculated as mg/g d.wt following calibration curve with bovine serum albumin protein.

Estimation of total free amino acids: Amino acid content was determined, 0.1 g dried callus was grinded in 5 ml of 80 % ethanol, filtered and centrifuged. To 0.1 ml of extract 1.9 ml of ninhydrin-citrate buffer-glycerol mixture was added[24]. The mixture was heated in boiling water bath for 12 min, cooled to room temperature and following shaking, the optical density was read at 570 nm within 1h. The blank was prepared 0.1 ml of 80 % ethanol instead of extract. Glycine was used for standardization. The amino acid concentration was expressed as mg per g dry mass.

Statistical analysis: The obtained results were statistically assessed using one and two way ANOVA to determine the degree of significance[25]. All statistical methods were applied using the SPSS statistical software package. Standard errors were added in the tables as ± SE.

RESULTS

The optimization of the *in vitro* production of secondary compounds of *Silybum marianum* L. tissue culture was carried out under laboratory conditions. Concentrations of the auxin-analogue 2,4-D, the cytokinin BAP, and myo-inositol were systematically altered and led to significant variations in the plant callus growth parameters (Table1).

Callus growth criteria

The growth parameters fresh weight and dry weight of the calli were recorded after 21, 42 and 63 d (Table 1). After 21 d on medium K which contained 0.5 mg/l 2,4-D combined with high concentration of Asn, BAP and inositol callus accumulated the highest amount of biomass(FW 120 and DW 18 mg) followed by medium N supplemented with high concentrations of all growth regulators. The lowest value of FW and DW was recorded on media H with 28 mg and 7 mg respectively. Thus a 4.6 times variation in callus FW and 2.5 fold variation in callus DW was achieved by changing the growth regulators concentrations. After 42 and 63 d medium F with a low concentration of 2,4-D combined with intermediate concentrations of Asn, BAP and inositol enabled the highest callus mass production. The lowest levels of callus biomass after 42 and 63 d was observed in medium H supplemented with 0.5 mg/l 2,4-D in the absence of Asn, BAP and inositol. The highest and lowest callus FW and DW differed by factors between 4.5 and13.3 times at 42 and 63 d respectively. The results indicate the predominant significance of adjusting the concentration of the auxin analogue.

Callus growth rates (Table 2) varied with progression of the experiment and in dependence of the 2,4-D concentration. Media supplemented with 0.5 mg/l 2,4-D combined with high concentration of Asn, BAP and inositol (medium K) produce the highest rate of callus growth after 21 d of 57.5 %. The highest growth rate was 12 % per day and occurred at low concentration of 2,4-D combined with medium concentrations of Asn, BAP and inositol (medium F) after 42 d. Medium I enabled the highest growth rate after 63 d which corresponded to 19 % per day. Media free of 2,4-D with intermediate and high concentration of Asn, BAP and inositol(media B, I and H) caused low growth rates after 21, 42 and 63 d respectively.

Callus volumes grown in high 2,4-D concentration combined with intermediate concentrations of Asn, BAP and inositol (medium N) produced the maximum volume after 21 d. After 42 d medium G induced the highest callus volume (0.4 cm^3).After 63 days 0.25 mg/l 2,4-D combined with medium concentrations of Asn, BAP and inositol (media F) produced callus volume 1.5cm^3. Media H, L and D produced the lowest volume of *S. marianum* callus after 21, 42 and 63 d.

Callus water content was strongly influenced by media composition in a time dependent manner (Table 3). The low concentration of 2,4-D (0.25 mg/l) with medium concentrations of Asn, BAP and inositol in medium F at 63 d gave the highest callus water content of 1225 %, followed by medium J with 0.5 mg/l 2,4-D and intermediate concentrations of Asn, BAP and inositol. Media free of 2,4-D with intermediate (medium B) and high concentrations (medium C) of Asn, BAP and inositol and media lacking Asn, BAP and inositol (medium H) led to the lowest callus water contents in the range of 500 % after 63 day. Media supplemented with 1 mg/l 2,4-D

combined with medium concentrations of Asn, BAP and inositol (medium M) produced high amount of callus water content after 21 d also in absence of Asn, BAP and inositol (medium L) produce the highest water content after 42 d. It is also important to note that the RWC of callus increased with time of callus growth on the different media.

Callus characteristics such as FW, DW, RWC and GR depended on the time of growth regardless of the medium growth regulator composition (Fig.1). While callus FW, DW and RWC increased with growth time as expected, the relative GR on the opposite decreased with time. These data indicated that the increase in callus FW was a result of increased RWC and to a minor extent of callus growth. This was confirmed by the increase in callus DW indicating accumulation of metabolites in callus.

Callus bulk density was the lowest (70 mg/cm^3) in medium J supplemented with 0.5 mg/l 2,4-D and intermediate concentrations of Asn, BAP and inositol after 63 d. Medium E produced the highest of 122 mg/cm^3. Medium B and F produced the highest bulk density after 21 and 42 d respectively.

Callus induction percentage and time

High callus induction required high concentrations of all growth regulators (Table 4) as confirmed by 100% callus induction in medium J and O. The lowest induction occurred in medium D containing lowest 2,4-D levels and lacking Asn, BAP and inositol. In media without Asn, BAP and inositol increased 2,4-D concentration enhanced callus induction with 86% callus induction in medium L. Media devoid of 2,4-D needed high concentration of the other growth regulators for (medium C 83% callus induction). Short periods of around 10 d required for callus induction are important characteristics for suitable media, and were observed for media F, G and L. In a converse manner cotyledons on medium C needed a period of more than four weeks (31 d) for callus induction. This was the longest lag phases among all tested media. Medium C contains the highest concentration of the other growth regulators but lacks 2,4-D. The data reveal medium J which induced 100 % callus with a slight delay (14 d) as one of the best media because it also required intermediate concentrations of all growth regulators.

The ability of callus to grow and survive after first and second cutting of callus into two pieces and re-culturing on new media with the same composition was intended to reveal information on callus stability and sensitivity to disturbance (Table4). All calli survived(zero death) after cutting at 21d, while three media proved to be less suitable for callus survival after cutting at 42 d, namely H, L and O. These media lack BAP, inositol and Asn or containing the highest levels of all effectors. On media H and O half of the calli dried and 17 % was dry on medium L.

The callus growth rate after first and second cuttings was lower than from the primary cotyledon explants which indicate that callus age reduced its growth rate on new media (Table 4). The data also show that callus initiation must be from cotyledon. Absence or low 2,4-D concentrations combined with intermediate or high concentrations of Asn, BAP and inositol led to the highest growth rate after first initiation of callus from callus cutting with a maximum rate on medium E.Callus growth rate after second callus cutting was particularly high in the absence of 2,4-D in media supplemented by high concentrations of Asn, BAP and inositol (C medium). In addition, the used concentration of 2,4-D without Asn, BAP and inositol also enabled moderate growth rate of callus after the first cutting but caused a negative growth rate in media O,H and L after second cutting coinciding with growth stagnation and death.

Accumulation of important metabolites

Calli that had been grown on the various media for 63 days, were analyzed for contents of some nitrogenous primary and for secondary metabolites (Table 5).The content of silymarin, protein and amino acids varied significantly in the callus produced by the different media. Absence of 2,4-D with high concentration of Asn, BAP and inositol (medium C) produce the highest amount of total soluble protein 35 mg/g dw while medium G produce the lowest amount of protein. Protein accumulation in *S. marianum* seeds was high (40 mg/g dw) compared with callus produced protein by all kinds of media. In a converse manner, calli kept at high concentration of 2,4-D in the absence of Asn, BAP and inositol in medium L accumulated free amino acids (5.7 mg/g)more than on other media. Calli on medium F produced low amount of amino acids even with absence or intermediate concentrations of Asn, BAP and inositol. *S. marianum* seeds accumulate the lowest amounts of amino acids compared with callus from all other media types. This may indicate to greater drainage of amino acids into protein synthesis in the seeds as indicated by the ratio of amino acids and protein.

The accumulation pattern of the secondary metabolites classified as silymarin differed completely from that of protein and amino acids since 0.5 mg/l 2,4-D combined with intermediate concentrations of Asn, BAP and inositol (medium J) enabled the synthesis of the highest amount of silymarin (26 mg/g) compared with other media. Also, medium J enhanced silymarin synthesis of 2.9

times in callus compared to seeds. Likewise, callus grown with medium G synthesized the lowest levels of silymarin. The seeds acquired the lowest content of silymarin compared to all of the used media. Medium G which produced the lowest silymarin in callus led to slightly high value than that of seeds. Out of previous data (Table 1 and 5) medium F led to the greatest amount of silymarin where 118g dry weight produced 2.659g silymarin in comparison with medium J which mediated accumulation half this amount. Therefore medium F was used for the next experiments.

Modification of media by elicitors

In the next step, further improvement was tested in the presence of low molecular mass antioxidants. To this end the growth criteria and callus induction efficiency were measured in the improved medium Fin the presence of different concentrations of glutathione and ascorbate (Table 6 and 7).

Effect of glutathione and ascorbate elicitor concentration on callus growth criteria

a-Glutathione: Cotyledons of *S. marianum* seeds were cultured on solidified MS F media containing 0, 0.1, 0.5, 1 or 2mM glutathione elicitor. Callus fresh weight was significantly increased by 0.1 mM glutathione (22 %comparing with the control) after 21d and with 26 % after 42d (Table 5). Then FW progressively decreased with increasing glutathione concentration and after 21 and 42 days. The dry weight of callus was increased slightly by 0.1mM glutathione but decreased progressively when concentration increased. Accordingly callus growth rate was highest at 0.1 mM after 21 d.

b- Ascorbate: With distinct concentration dependency than glutathione, ascorbate had a positive effect on callus fresh weight with increasing the concentration up to 1 mM where the increase was 35% after 21d, while only 5% at 100 µM after 42d and it tended to decrease at 2 mM ascorbate. The dry weight on the opposite decreased with an increasing amount by the ascorbate concentrations indicating that the increase in fresh weight was due to increased water content. Callus growth rate significantly increased with increasing ascorbate concentration up to 1 mM after 21d, while it tended to decrease with increasing concentration of ascorbate after 42d.

Effect of glutathione and ascorbate on callus induction

Percentage of callus induction is an important parameter for biomass production. It reached 100 % at low glutathione concentrations, but slightly dropped at 1 and 2 mM where induction was 83% and 88% respectively. Different concentrations of ascorbate increased callus induction to 100 % compared with the control of plain medium F which was 83 %.

Effect of glutathione and ascorbate elicitor concentration on callus silymarin

The presence of glutathione and ascorbate elicitors altered the accumulation of silymarin in *S. marianum* callus after 42 d (Figure2). Glutathione supplementation to the medium F increased accumulation of silymarin with a maximum at 0.1mM (by 10.5%) and reduced it at higher concentrations. Supplementation of medium F with different ascorbate concentrations had no significant impacts on silymarin biosynthesis even higher concentrations more than 0.1mM inhibited it remarkably.

Effect of culture age on accumulation of silymarin in the presence of glutathione and ascorbate

Results of silymarin contents in callus after adding glutathione and ascorbate for short period were recorded in (Fig. 3). Callus with age 42 day were sub-cultured on medium F in the presence of glutathione and ascorbate. Callus was harvested after short time periods of 4, 8, 12 and 16d.The silymarin content depended on the glutathione concentration. After 4 d silymarin content was only different in medium supplemented with 0.1mM glutathione where it had increased by 4.6% compared to F medium. After 8 d silymarin content was highest in the presence of 1 mM glutathione with 27.8% over control. Callus harvested from media supplemented with different ascorbate concentrations revealed no significant differences in silymarin accumulation. Maximum silymarin accumulation occurred in callus with 0.5mM ascorbate after 8d harvest at which initiate silymarin was by 20.8%.

Effect of phenylalanine precursor on silymarin accumulation

The amount of silymarin in callus tissue varied due to the increase in phenylalanine concentration in medium F (Fig.4). Silymarin increased in phenylalanine- supplemented medium with a maximum at 25 µM which enhanced accumulation of silymarin significantly by 31.4%. Silymarin accumulation decreased with increasing the concentration of phenylalanine.

Accumulation of silymarin in media with different modifications

The silymarin contents on the different modifications of F medium are summarized in (Fig.5). Media F was

already selected from 14 tested media and used as control for comparison between different modifications. Addition of 25µM phenylalanine,as a precursor and 1mM of glutathione after 8 d and 0.5 mM of ascorbate after 8 d as elicitors to media F induced callus increased silymarin accumulation by 31.4, 6.4 and 30.6% respectively, compared with that induced by media F. Apparently supplementation of media F with phenylalanine is particularly suited to increase the induced callus mass and its silymarin content.

DISCUSSION

S. marianum callus may serve as alternative source for liver protective pharmacological drugs and thus callus-based approaches would be of significant interest for economic and ecological reasons[26-27]. Callus induction from cotyledons in the present study was strongly affected by endogenous and exogenous factors. Supplementing the growth media with different concentrations of mineral elements, growth regulators, amino acids and vitamins is known to influence callus induction and accumulation its secondary metabolites[28-29-30]. The auxin-analogue 2,4-D and the cytokinin 6-BAP as growth substances in the used concentrations acquired varied effects on callus formation. The use of 2,4-D alone or in combination with other hormones has become almost routine in inducing somatic embryogenesis in seed culture [31]. It is often used despite indications that 2,4-D might cause some health problems [32]. Therefore, keeping 2,4-D at least needed level is intended in this study.

After 21 d of callus growth medium K containing 0.5 mg/l 2,4-D with high concentrations of Asn, BAP and inositol gave rise to the highest amount of callus biomass (FW 120 mg and DW 18 mg) followed by medium N supplemented with high concentrations of all growth regulators as also found[33-34]. The lowest value of FW 28 mg and DW 7 mg occurred in medium H supplemented by 0.5 mg/l 2,4-D in the absence of Asn, BAP and inositol. Out of the present results, the suitable growth regulator concentrations combination of medium F resulted in 4.6and 2.5 times elevated callus FW and DW. After 42 and 63 d of callus growth on medium F with low 2,4-D concentration combined with intermediate concentrations of Asn, BAP and inositol caused the highest callus FW and DW while medium H still caused the lowest of them.

Increasing time of callus growth increased the difference between the highest and lowest callus FW and DW which were 8.3, 13.3 and 5.4 times at 42 and 63 d of callus growth respectively. This specifies the significance of appropriately adjusting the concentration of the growth regulators and the time for harvesting the best callus mass[35]. It is also important to note that the RWC of callus increased with time of callus growth under the different media. Callus growth rate was enhanced by low 2,4-D concentration (media F and I) but it was reduced to its minimum in media free of it (media B, I and H)[36-37]. The calculated relationships indicated an increasing trend of callus FW, DW and RWC with time of callus growth while GR on the opposite showed a decreasing trend of variation because of the increase in callus DW and water content caused by accumulation of metabolites in callus and not exhausted in growth activity. This was confirmed by the increase in Callus bulk density from 70 to 122 mg/cm^3 indicating appreciable effect of medium adjustment on dry mass accumulation in callus.

Physical and chemical stimuli have been widely employed as elicitors to increase desired compounds in plant cell cultures such as terpenoids[38], coumarin derivatives[39], alkaloids[40] and flavonoids [41]. Elicitation is also effective on a secondary pathway which leads to the production of interested compounds which are not phytoalexins[38].

There is a growing body of evidence indicating that the role of ascorbate and glutathione in plants extends beyond their intensively explored antioxidant function[42]. It has been demonstrated that high ascorbate and glutathione levels are required for normal progression of the cell cycle in meristematic tissues[43-44-45-46-47]. Present results also showed significantly increases in callus fresh and dry weights by 0.1 mM glutathione (22 % after 21d and 26 % after 42d.) while high concentrations progressively decreased callus weights at both dates of callus growth. Callus growth rate reached its highest percentage at 0.1mM after 21 d.

Glutathione has been considered as a hub in cell redox metabolism due to its multiple and central functions. Glutathione is buffer of the thiol redox state of cellular compartments, protects and regulates protein thiols by glutathionylation, is regenerating substrate of ascorbate in the water-water cycle, stores nitric oxide as nitrosoglutathione, is conjugated to xenobiotics and metabolites for transport and compartmentation and donates electrons via glutaredoxins to peroxiredoxins which are involved in peroxide detoxification and redox signaling[48-49-50]. Modifications in cell glutathione contents alter gene expression and metabolism, e.g. induces expression of defense genes[51]. Thus it is not surprising that supplementation of callus medium with glutathione alters synthetic activities for secondary compounds as shown here for silymarin.

Ascorbate concentrations up to 1 mM after 21d and to 100 µM in 42d callus had a positive effect on callus fresh weight with increases by 35% and 5% respectively. Higher concentration decreased callus FW. Dry weight on the opposite decreased with an increasing ascorbate concentrations indicating that the increase in fresh weight was due to increased water content. Callus induction was 100 % at all concentrations of ascorbate and glutathione except high concentrations of glutathione. Ascorbate is directly involved in the regulation of two processes that mediate morphogenic responses in plants: cell division and elongation in addition to the regulation of mitotic activity in the meristems[52-53-54].

Phenylalanine as precursor of flavonoids caused a marked silymarin accumulation. Phenylalanine at 25 µM concentration enhanced accumulation of silymarin by 31.4% as found by many author's [55-56-57]. The exogenous addition of phenylalanine or intermediate compounds to culture medium may increase the yield of compounds of interest in *Taxus* cultures[17].

In summary, the conditional accumulation of silymarin in callus of *S. marianum* was a result of growth regulators and time of callus harvesting adjustment. It is also, a function of phenylalanine as precursor, glutathione and ascorbate which should be further explored in combination. This should allow for maximizing the yield of callus biomass production and accumulation of secondary compounds with potential effects in liver disease treatment.

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Table 1

Effect of medium composition on *S. marianum* callus fresh weight (FW) and dry weight after 21, 42 and 63 days of growth.

					FW [mg]			DW [mg]		
Growth regulators levels [mg/l]					21 d	42 d	63 d	21d	42 d	63d
Media Code	2,4-D	Asn	BAP	Inositol						
A	0	25	0.01	25	NI	NI	NI	NI	NI	NI
B	0	50	0.05	50	35±7^{a}	54±10^{a}	98±12^{a}	12±2^{bcde}	10±1^{a}	15±0.5^{a}
C	0	100	0.1	100	51±19^{ab}	72±12^{a}	139±3^{a}	13±3^{bcde}	15±2^{ab}	23±1^{ab}
D	0.25	0	0	0	29±8^{a}	52±9^{a}	92±14^{a}	9±1^{ab}	11±1^{a}	13±1^{a}
E	0.25	25	0.01	25	26±8^{a}	61±15^{a}	175±84^{a}	8±1^{a}	12±2^{a}	22±7^{ab}
F	0.25	50	0.05	50	79±13^{bcd}	347±78^{c}	1565±227^{e}	12±2^{bcde}	45±9^{d}	118±11^{f}
G	0.25	100	0.1	100	97±17^{cde}	283±46^{c}	738±66^{cd}	14±2^{def}	35±4^{c}	60±7^{d}
H	0.5	0	0	0	28±5^{a}	47±8^{a}	75±20^{a}	7±1^{a}	11±1^{a}	14±2^{a}
I	0.5	25	0.01	25	75±25^{bcd}	75±25^{a}	929±282^{d}	15±1^{ef}	14±3^{ab}	88±16^{e}
J	0.5	50	0.05	50	78±10^{bcd}	125±17^{ab}	743±237^{cd}	13±1^{cde}	19±2^{ab}	56±13^{cd}
K	0.5	100	0.1	100	120±10^{e}	126±24^{ab}	391±74^{abc}	18±1^{f}	19±3^{ab}	38±4^{bc}
L	1	0	0	0	47±11^{ab}	90±12^{ab}	119±26^{a}	8±1^{a}	11±1^{a}	14±2^{a}
M	1	25	0.01	25	61±8^{abc}	66±11^{a}	287±68^{ab}	11±1^{abcd}	13±1^{a}	31±6^{ab}
N	1	50	0.05	50	98±13de	167±38^{b}	559±100^{bc}	13±1^{bcde}	23±4^{b}	53±7^{cd}
O	1	100	0.1	100	61±14^{abcd}	97±15^{a}	123±20^{a}	10±1^{abc}	12±1^{a}	18±2^{ab}
F- Value					*5.00*	*9.40*	*12.08*	*5.05*	*10.5*	*18.34*
Probability					*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*

Each value represents the mean ± standard error, analysed by ANOVA test and Fisher LSD. Different letters in the same column indicate significance of difference at P≤0.05. No induction: NI.

Table 2
Effect of medium composition on *S. marianum* callus growth rate (GR) and callus volume (V) after 21, 42 and 63 days of growth

					GR[% d^{-1}]			V [cm^3]		
	Growth regulators levels [mg/l]				21day	42day	63day	21 day	42 day	63 day
Media code	2,4-D	Asn	BAP	Inositol						
A	0	25	0.01	25	NI	NI	NI	NI	NI	NI
B	0	50	0.05	50	15±3^{ab}	3±0.3^{a}	3.8±0.7^{ab}	0.08±0.01^{a}	0.18±0.03^{a}	0.166±0.02^{a}
C	0	100	0.1	100	16±8^{ab}	8±1.0^{ab}	3.3±1.8^{abc}	0.09±0.02^{ab}	0.15±0.03^{a}	0.25±0.03^{ab}
D	0.25	0	0	0	12±1^{a}	4±0.6^{a}	3.7±0.6^{ab}	0.08±0.01^{a}	0.15±0.02^{a}	0.15±0.02^{a}
E	0.25	25	0.01	25	17±9^{ab}	8±0.7^{ab}	4.4±0.5^{abcd}	0.09±0.02^{ab}	0.16±0.04^{a}	0.183±0.04^{a}
F	0.25	50	0.05	50	50±12de	12±0.3^{f}	10.8±2.3^{f}	0.13±0.02^{abcd}	0.38±0.07^{c}	1.53±0.2^{f}
G	0.25	100	0.1	100	48±13^{cde}	10±1.5^{ef}	7.7±1.5^{e}	0.17±0.02^{cdef}	0.40±0.07^{c}	0.6±0.06^{cd}
H	0.5	0	0	0	16±6^{ab}	5±0.7^{ab}	-0.5±0.4^{a}	0.08±0.01^{a}	0.12±0.02^{a}	0.2±0.04^{a}
I	0.5	25	0.01	25	37±14^{bcde}	1±1.8^{ab}	19.1±3.0^{de}	0.20±0.00^{ef}	0.14±0.04^{a}	0.966±0.2^{e}
J	0.5	50	0.05	50	53±7de	2±0.3^{abcd}	2.7±0.5^{abcd}	0.20±0.03^{ef}	0.22±0.02^{ab}	0.8±0.2^{de}
K	0.5	100	0.1	100	58±6^{e}	5±0.8^{cde}	5.0±3.2^{cde}	0.18±0.02^{def}	0.17±0.03^{a}	0.45±0.03^{abc}
L	1	0	0	0	25±4^{abc}	4±0.2^{ab}	-1.1±0.4^{ab}	0.15±0.02^{bcde}	0.13±0.02^{a}	0.166±0.03^{a}
M	1	25	0.01	25	39±5^{bcde}	10±1.3^{de}	2.8±0.5^{bcd}	0.13±0.03^{abcd}	0.13±0.02^{a}	0.333±0.05^{abc}
N	1	50	0.05	50	57±9^{e}	2±0.7^{bcd}	16.8±5.6^{e}	0.22±0.03^{f}	0.30±0.04^{bc}	0.566±0.07^{bcd}
O	1	100	0.1	100	33±11^{abcd}	4±0.3^{abc}	3.9±1.2^{abcd}	0.12±0.03^{abc}	0.18±0.03^{a}	0.166±0.02^{a}
F- Value					*3.9*	*4.1*	*11.1*	*5.5*	*6.0*	*11.3*
Probability					*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*

. Each value represents the mean ± standard error, analysed by ANOVA test and Fisher LSD. Different letters in the same column indicate significance of difference at P≤0.05. No induction: NI.

Table3
Effect of medium composition on *S. marianum* callus relative water content (RWC) and callus bulk density (CBD) after 21, 42 and 63 days of growth.

					RWC [%]			CBD [mg/cm^3]		
	Growth regulators levels [mg/l]				21day	42day	63day	21 day	42 day	63 day
Media code	2,4-D	Asn	BAP	Inositol						
A	0	25	0.01	25	NI	NI	NI	NI	NI	NI
B	0	50	0.05	50	193±22^{a}	434±70^{abc}	568±62^{abc}	158±15^{g}	55±9^{a}	88±13^{ab}
C	0	100	0.1	100	307±42^{ab}	373±27^{a}	513±114^{ab}	136±14^{fg}	101±16de	91±8^{ab}
D	0.25	0	0	0	211±43^{a}	357±44^{a}	588±62^{abc}	112±12^{ef}	76±9^{abc}	89±15^{ab}
E	0.25	25	0.01	25	249±65^{ab}	421±47^{ab}	684±79^{abc}	82±20^{cde}	74±16^{abcde}	122±13^{ab}
F	0.25	50	0.05	50	545±44de	681±44^{def}	1225±83^{g}	91±5^{bcde}	116±15^{e}	77±12^{ab}
G	0.25	100	0.1	100	584±72de	708±65^{ef}	1133±39^{fg}	85±10^{abcde}	88±4^{abcde}	100±11^{ab}
H	0.5	0	0	0	305±49^{abc}	321±44^{a}	453±57^{a}	84±10^{bcde}	96±9^{bcde}	68±19^{ab}
I	0.5	25	0.01	25	386±160^{bcd}	452±57^{ab}	956±189de	77±5^{abcd}	95±12^{cde}	91±54^{b}
J	0.5	50	0.05	50	483±44^{cd}	552±25^{bcd}	1223±148^{efg}	67±7^{abc}	88±4^{abcde}	70±8^{a}
K	0.5	100	0.1	100	566±41de	580±35^{cde}	920±94^{def}	98±13de	111±9^{e}	85±3^{ab}
L	1	0	0	0	502±87^{cd}	718±110^{f}	757±66^{bcd}	52±4^{a}	83±8^{abcd}	83±15^{ab}
M	1	25	0.01	25	479±61^{cd}	395±41^{a}	840±82^{cd}	84±16^{cde}	100±11^{cde}	92±9^{ab}
N	1	50	0.05	50	685±59^{e}	641±52^{def}	962±58^{defg}	58±5^{ab}	75±6^{ab}	93±9^{ab}
O	1	100	0.1	100	524±66^{cd}	684±78^{def}	583±87^{abc}	84±11^{cde}	67±12^{abc}	108±14^{ab}
F- Value					*5.13*	*7.10*	*7.06*	*6.6*	*2.5*	*0.73*
Probability					*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.72*

Each value represents the mean ± standard error, analysed by ANOVA test and Fisher LSD. Different letters in the same column indicate significance of difference at P≤0.05. No induction: NI.

Table 4

Effect of media composition on percentage of callus induction (PCI), day until callus induction (DCI), mortality of callus after first cutting (MFC), mortality after second cutting (MSC), growth rate after first cutting (GRFC) and growth rate after second cutting (GRSC).

	Growth regulators level [mg/l]				PCI [%]	DCI [day]	MFC [%]	MSC [%]	GRFC [% d^{-1}]	GRSC [% d^{-1}]
Media Code	**2,4-D**	**Asn**	**BAP**	**Inositol**						
A	0	25	0.01	25	NI	NI	NI	NI	NI	NI
B	0	50	0.05	50	62±5^{b}	21±0.6^{f}	0±0	0±0^{a}	10±1^{abc}	0.3±0.1^{a}
C	0	100	0.1	100	83±1^{cd}	31±1.5^{g}	0±0	0±0^{a}	13±1^{c}	5.5±1^{e}
D	0.25	0	0	0	28±1^{a}	18±1.2^{de}	0±0	0±0^{a}	6±1^{abc}	0.1±0.1^{a}
E	0.25	25	0.01	25	63±2^{b}	19±1.2^{ef}	0±0	0±0^{a}	16±9^{d}	1.4±0.6^{abc}
F	0.25	50	0.05	50	83±1^{cd}	10±1.2^{a}	0±0	0±0^{a}	12±1^{bc}	3.4±0.7^{d}
G	0.25	100	0.1	100	97±1^{f}	10±0.6^{a}	0±0	0±0^{a}	5±0.6^{ab}	0.8±0.1^{a}
H	0.5	0	0	0	74±3^{c}	16±1.0^{bcd}	0±0	50±3^{c}	8±3^{abc}	-0.2±0.3^{a}
I	0.5	25	0.01	25	60±7^{b}	15±0.0^{bc}	0±0	0±0^{a}	7±1^{abc}	3.1±1^{cd}
J	0.5	50	0.05	50	100±0^{f}	14±0.6^{b}	0±0	0±0^{a}	3±0.6^{a}	3.2±0.9^{cd}
K	0.5	100	0.1	100	99±1^{f}	14±0.0^{b}	0±0	0±0^{a}	5±0.9^{ab}	3±0.9^{bcd}
L	1	0	0	0	86±2de	10±0.6^{a}	0±0	17±0.7^{a}	6±1^{ab}	-0.5±0.4^{a}
M	1	25	0.01	25	86±3de	14±0.6^{b}	0±0	0±0^{a}	4±1^{ab}	1.1±0.2^{abc}
N	1	50	0.05	50	92±2^{ef}	15±0.0^{bc}	0±0	0±0^{a}	7±1^{abc}	0.8±0.3^{ab}
O	1	100	0.1	100	100±0^{f}	17±0.6^{cde}	0±0	50±2^{c}	3±0.5^{a}	-0.7±0.7^{a}
Statistical analysis										
F- Value					*52.3*	*46.0*	*0.0*	*351.6*	*3.6*	*6.8*
Probability					*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*

Each value represents the mean ± SE and is assigned to statistical groups byANOVA test and Fisher LSD. Similar letters in the same column are not significantly varied at P≤0.05. No induction: NI.

Table 5

Effect of medium concentrations on callus constituents silymarin, total soluble protein, amino acids and amino acids/protein ratio after 63 day of callus growth.

	Growth regulators level mg/L				Soluble Protein [mg/g dw]	Amino acids [mg/g dw]	Amino acids/Protein ratio
Media codes	**2,4-D**	**Asn**	**BAP**	**Inositol**			
A	0	25	0.01	25	NI	NI	NI
B	0	50	0.05	50	25±1.5^{c}	3.6±0.14^{ab}	0.141±0.01^{abc}
C	0	100	0.1	100	35±0.7^{f}	5.0±0.33^{efg}	0.144±0.01^{abc}
D	0.25	0	0	0	24±1.6^{c}	3.6±0.10^{ab}	0.152±0.01^{bcd}
E	0.25	25	0.01	25	33±0.4^{ef}	4.9±0.14^{efg}	0.151±0.0^{bcd}
F	0.25	50	0.05	50	26±1.3^{cd}	3.2±0.08^{a}	0.120±0.0^{a}
G	0.25	100	0.1	100	12±1.0^{a}	4.4±0.14^{cd}	0.360±0.02^{h}
H	0.5	0	0	0	32±1.2^{e}	4.0±0.09^{bc}	0.127±0.01^{ab}
I	0.5	25	0.01	25	34±0.7^{f}	5.1±0.12^{fg}	0.147±0.0^{abcd}
J	0.5	50	0.05	50	24±1.2^{c}	3.8±0.11^{b}	0.157±0.0^{cde}
K	0.5	100	0.1	100	25±0.4^{c}	4.7±0.31^{def}	0.184±0.01^{e}
L	1	0	0	0	26±0.3^{c}	5.7±0.32^{h}	0.220±0.01^{f}
M	1	25	0.01	25	18±0.6^{b}	4.5±0.14^{cde}	0.246±0.01^{g}
N	1	50	0.05	50	29±0.4^{d}	5.0±0.17^{efg}	0.174±0.01^{de}
O	1	100	0.1	100	21±0.8^{b}	5.4±0.08^{gh}	0.260±0.01^{g}
Seeds	0	0	0	0	40±0.9	2.5±0.19	0.063±0.01
Statistical analysis							
F-value					*55.4*	*23.2*	*58.2*
Probability					*0.00*	*0.00*	*0.00*

Each value represents the mean ± SE and is assigned to statistical groups by ANOVA test and fisher LSD. Similar letters in the same column are not significantly varied at P≤0.05. No callus induction (NI).

Table 6

Effect of different concentrations of glutathione and ascorbic acid on growth criteria of *S. marianum* callus, fresh weight (FW), dry weight (DW), relative water content (RWC), growth rate (GR), percentage of callus induction (PCI) and days until callus induction (DCI) after 21 days.

21 days							
Elicitors	**Conc. [mM]**	**FW [mg]**	**DW [mg]**	**RWC [%]**	**GR[% d^{-1}]**	**PCI [%]**	**DCI [day]**
Glutathione	**0.0**	402±24 b	37±4 b	984±48 bc	223±14 b	100±0 b	7±0.5 a
	0.1	515±18 c	38±2 b	1248±66 c	287±10 c	100±0 b	7±0.5 a
	0.5	179±22 a	24±6 a	697±198 ab	84±13 a	100±0 b	7±0.5 a
	1.0	113±7 a	16±2 a	589±34 a	60±4 a	83±6 a	7±0.5 a
	2.0	132±14 a	17±2 a	655±81 a	70±8 a	88±3 a	7±0.5 a
Ascorbate	**0.0**	271±15 a	29±1 ab	821±56 a	149±9 a	100±0 a	7±0.5 a
	0.1	390±12 b	29±1 ab	1236±82 b	216±7 b	100±0 a	7±0.5 a
	0.5	360±21 b	28±2 ab	1203±129 b	199±12 b	100±0 a	7±0.5 a
	1.0	419±23 b	32±2 b	1220±77 b	233±13 b	100±0 a	7±0.5 a
	2.0	296±31 a	26±2 a	1027±81 ab	163±17 a	100±0 a	7±0.5 a

Each value represents the mean ± standard error from XY experiments. Same letters in the same column indicate lack of significant at P≤0.05.

Table 7

Effect of different concentrations of glutathione and ascorbic acid on contents of silymarin and on growth criteria of *S. marianum* callus, fresh weight (FW), dry weight (DW), relative water content (RWC) and growth rate (GR) after 42 days.

42 days						
Elicitors	**Conc. [mM]**	**FW [mg]**	**DW [mg]**	**RWC [%]**	**GR[% d^{-1}]**	**Silymarin [mg/g]**
Glutathione	**0.0**	737±61 b	64±5 b	1057±49 ab	4.0±1.3 a	19±2.1ab
	0.1	857±104 b	65±5 b	1225±130 b	3.2±1.1 a	21±0.4b
	0.5	156±26 a	20±2 a	636±96 a	0.70±0.8 a	19±1.6ab
	1.0	146±38 a	18±3 a	728±352 ab	1.4±1.9 a	17±2.7a
	2.0	191±17 a	22±1 a	757±64 ab	2.1±1.1 a	17±2.7a
Ascorbate	**0.0**	646±40 b	48±3 a	1249±115 a	6.6±1 c	26±3a
	0.1	686±43 b	47±3 a	1365±105 a	3.6±0.5 b	26±3a
	0.5	461±64 a	41±4 a	1036±141 a	1.3±1 ab	22±3a
	1.0	489±56 a	39±4 a	1144±150 a	0.8±0.4 a	25±4a
	2.0	491±34 a	44±3 a	1027±111 a	3.1±0.5 b	26±2a

Each value represents the mean ± standard error from XY experiments. Same letters in the same column indicate lack of significant at P≤0.05.

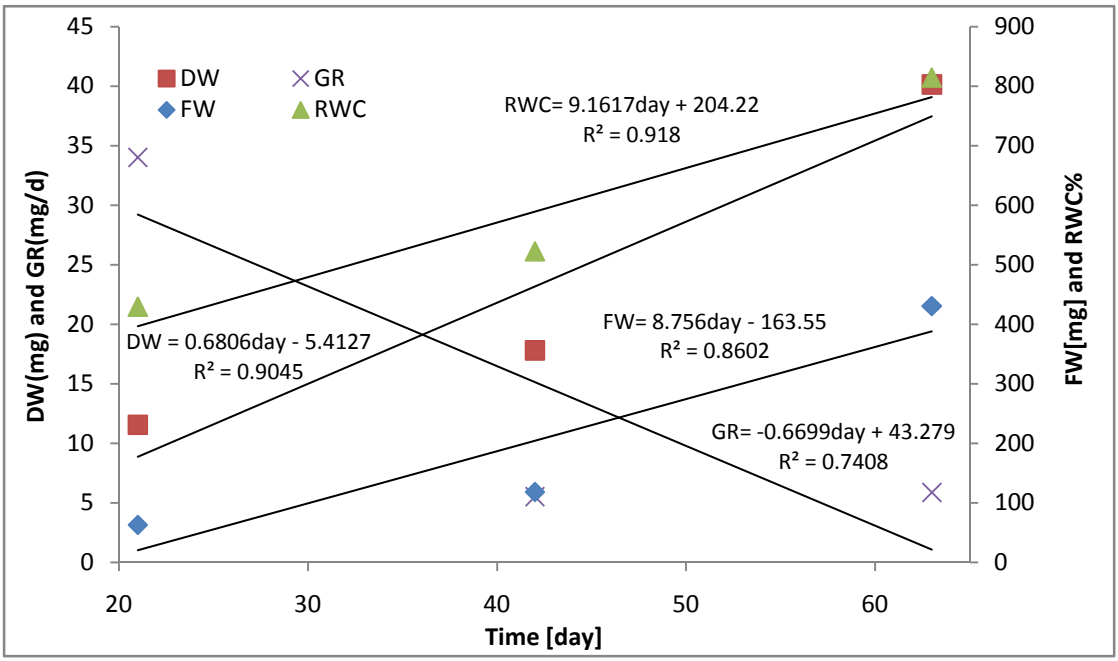

Fig 1
The dependency of callus FW, DW, RWC and GR on time of growth regardless of the medium growth regulator composition indicated significant linear relationships.

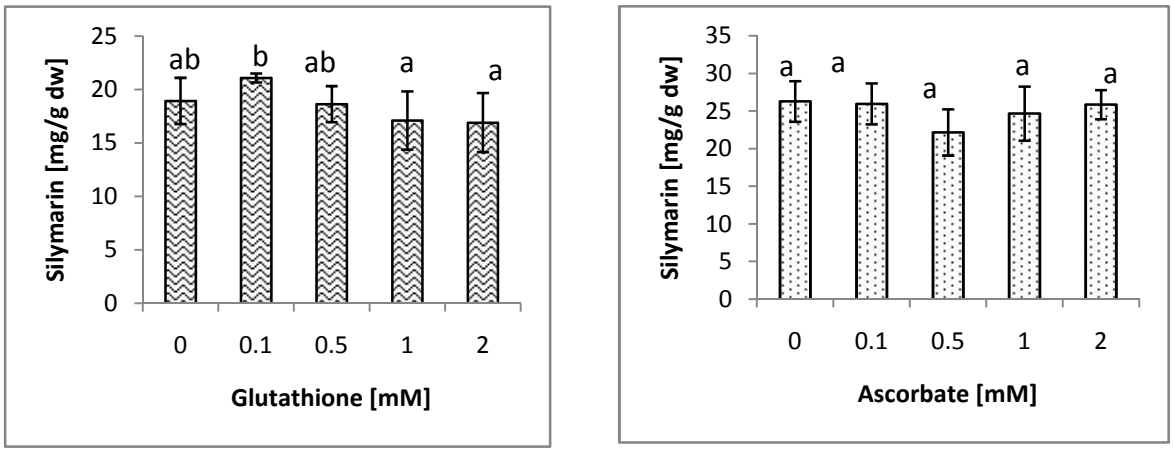

Fig 2
Effect of glutathione and ascorbate supplementation for 42 d on contents of silymarin in *S. marianum* callus tissue (cf. table 7).

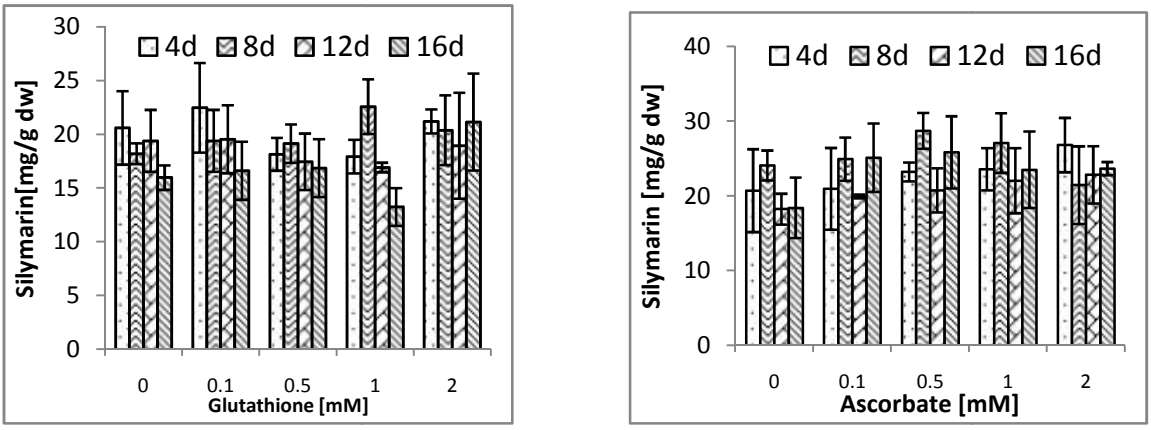

Fig 3

Effect of adding different concentrations of glutathione and ascorbate in callus media for short periods (4, 8, 12 and 16 day) on silymarin content in callus tissue.

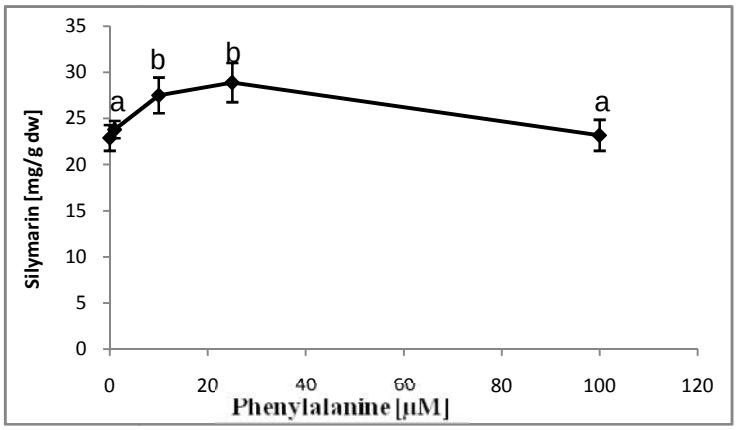

Fig 4

Effect of adding different concentrations of phenylalanine to the media on silymarin accumulation in *S. marianum* callus tissue.

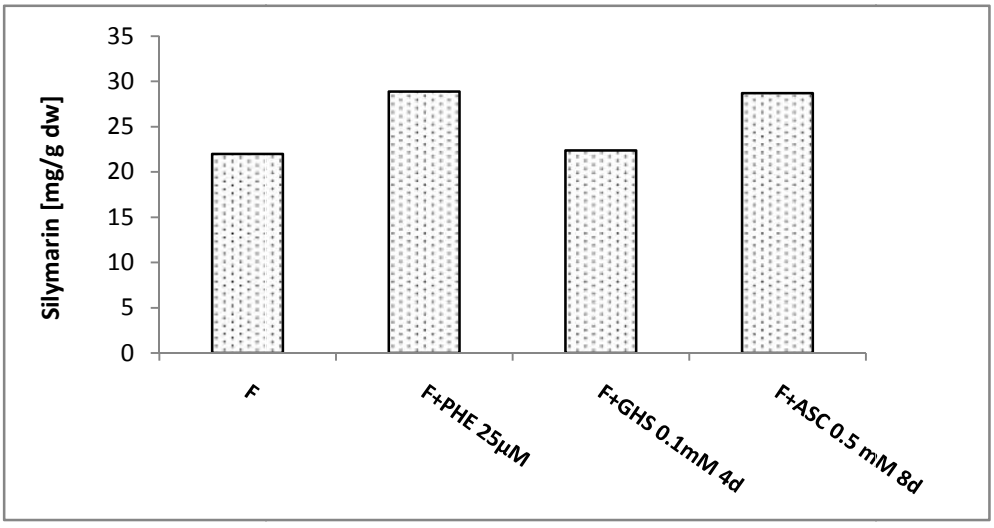

Fig 5

Comparison between silymarin with different modifications

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