



Evaluation of thermal-stress on the accumulation of podophyllotoxin in shoot *in vitro* cultures of *Linum persicum*

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Abstract

Linum persicum is an endangered plant native to Iran, from Linaceae family. Phytochemical analysis has shown that *L. persicum in vitro* cultures contain podophyllotoxin (PTOX), the pharmacological precursor for anticancer drugs such as etoposide, etopophos, and teniposide. Shoot culture of *L. persicum* was established from callus cultures in MS medium containing GA₃. A study was designed to investigate the effects of thermal-stress treatments (TST) on the shoot growth pattern of *L. persicum* along with the accumulation of PTOX. Shoots were heat-stressed at 37, 40, 45, 50, and 55 °C for 3 h, and then transferred back to the growth room. In each thermal group after 2, 3, and 4 weeks, 4 flasks were extracted for PTOX analysis. HPLC analysis ($p \leq 0.05$) revealed that shoots which had experienced 37 °C of TST after 4 weeks showed the highest amount of growth as well as PTOX production, 1.62±0.21% dry weight.

Keywords: *Linum persicum*, podophyllotoxin, shoot culture, thermal stress

Introduction

Podophyllotoxin (PTOX) is a naturally-occurring aryl-tetralin lignan, particularly found in the genus *Podophyllum* [1], *Linum* [2], *Juniperus* [3] and *Callitris* [4].

PTOX has important cytotoxic effects due to its microtubule inhibition properties [5]. Etoposide, teniposide, and etopophos are its semisynthetic derivatives which are used as chemotherapeutic agents in the treatment of testicular cancer, small cell lung cancer and certain tumors [6]. Rhizomes of *Podophyllum hexandrum* and *P. peltatum* are traditional sources of PTOX, which are endangered as a result of over harvesting [7]. Because of its complex structure, chemical

synthesis of PTOX is not economically feasible [8].

Tissue cultures of *Linum persicum* (Linaceae), which is called “Katan-e-Parsi” in Persian language, an endangered plant native to Iran [9], provide the opportunity to produce PTOX under controlled and reproducible conditions [10]. Optimization of culture process could improve product yield in plant tissue culture [11]. For instance changing cultivation parameters like pH, lighting condition, and temperature could result in raised plant growth rate and lignan accumulation. The average constant growth room temperature which has been reported in the

literature is 25 °C (with a range of 17-32 °C). In order to speed up growth and morphogenesis *in vitro*, this temperature is usually higher than what would be experienced by the same plants growing *in vivo* [12]. As reported in the literature, high-temperature stress would induce production of phenylpropanoids as a response to heat stress [13]. Therefore, this study was designed to investigate the effects of different thermal-stress treatments (TST) on the accumulation of PTOX and shoot growth in *in vitro* cultures of *L. persicum*.

Experimental

Plant material

Seeds of *L. persicum* Ky. ex Boiss. (Linaceae) were collected from Denna Mountain, about 40 km NW Yassuj, Iran at an altitude of 2750 m in August 2002. The species was identified by I. Mehregan and the voucher specimens were deposited at Shiraz Faculty of Pharmacy Herbarium (no. 229).

Shoot formation from callus cultures

Seed germination and callus initiation were carried out as was described previously [10]. After callus formation and establishment by subsequent culture passages in Murashige and Skoog (MS) basal media [14], supplemented with α -naphthalene acetic acid (α -NAA) 1 mg/mL, 2,4-dichlorophenoxyacetic acid (2,4-D) 0.5 mg/mL and Kinetin (Kn) 0.5 mg/mL, 3 g green callus tissues were transferred to MS medium supplemented with gibberellic acid (GA₃) 2 mg/mL for shoot initiation. All media contained 3% sucrose and 0.8% agar; pH was adjusted to 5.6 with 1N NaOH before autoclaving (1.2 kg/cm² and 121 °C for 20 min). Proliferated shoots were subcultured on the fresh media while callus or unorganized tissues were removed. The establishment of shoot culture was performed by 10 culture passages (3 weeks each one) on the same media and was incubated under continuous lightness (*ca* 2700 lux) and 27±2 °C.

Effect of thermal-stress on shoot formation and lignan accumulation

Four shoots with *ca* 1.5 cm length were cut and transferred to a 250 mL flask containing 50 mL MS medium supplemented with GA₃ 2 mg/mL (figure 1). 24 h after subculture, flasks were transferred to a thermally isolated cabin (TIC) which was designed for this study. Fluorescent lights were used in the cabin to create continuous lightness just like the growth room (*ca* 2700 lux). A digital thermometer was placed in the cabin and the temperature changes were recorded during the treatment. The flasks experienced 37, 40, 45, 50, and 55 °C of thermal-stress for 3 h. After TST, flasks were transferred back to the growth room. In each thermal group 4 flasks after 2 weeks, 4 flasks after 3 weeks, and 4 flasks after 4 weeks (12 flasks in each temperature) were extracted for PTOX analysis. After subculture, 12 flasks were remained at growth room (27±2 °C) and after 2, 3, and 4 weeks each time 4 flasks were extracted for PTOX analysis as the control groups. In order to record the temperature profile in addition to the growth room and the inner TIC, the temperature profiles of the inner of the flasks after transferring from growth room to TIC, and the inner of the flasks after transferring back from TIC to the growth room, were recorded by installing a digital thermometer in the flasks containing plantlet. Sample flasks were transferred to the TIC with the temperature of 43 °C and after 4 h, the flasks were transferred back to the growth room and the temperature changes were recorded (figure 2). To determine the shoot proliferation efficacy of TST, the mean number of leaves, the mean length of the shoots, and the total dry weight (DW) were recorded. For all parameters one-way analysis of variance (ANOVA) with tukey post Hoc test was applied with a critical value of $p \leq 0.05$.

Podophyllotoxin extraction and analysis

Lignan extraction was carried out as described by Emt et al. [15]. Samples were transferred to freezer (-20 °C) to inhibit their enzymatic activities. When their intercellular liquid was

frozen, they were lyophilized by freeze dryer for 24 h. Then their DW was measured and they were powdered to a very fine powder. 100 mg of the fine powder of lyophilized *in vitro* cultures was extracted with 1 mL ethanol in an ultrasonic bath (twice for 30 s with cooling on ice for 90 s between). Distilled water (3 mL) and 0.5 mg of β -glucosidase were added. pH was adjusted to 5.0 by *O*-phosphoric acid. Then, samples were incubated at 35 °C for 1 h. Ethanol (6 mL) was added and the mixture was incubated for 10 min at 70 °C in an ultrasonic bath. After centrifugation on 5000 r.p.m. for 10 min, the supernatant was used for HPLC analysis. During extraction, cells and solvents were mixed by using vortex. The HPLC analysis was performed in a Shimadzu instrument equipped with a UV detector in 290 nm, a column of 250 mm long and 4.6 mm inner diameter filled with Nucleosil 100 C₁₈, 5 μ m (particle size). The flow rate of the mobile phase was 1 mL/min and an isocratic system was applied with combination of water and acetonitrile as the eluent by the ratio of 55:45. PTOX was quantified according to commercial podophyllotoxin (Roth 3946.1). One-way analysis of variance (ANOVA) with tukey post Hoc test and $p \leq 0.05$ was conducted with PASW Statistics 18 for data analysis.

Results and Discussion

Shoot cultures of *L. persicum* were established from callus cultures (figure 1). Effects of TST on the accumulation of PTOX and shoot growth were studied. A TIC was designed and the flasks undergone TST in the cabin. In order to adjust TST, temperature of the inner flask environment along with the temperature of the cabin and the growth room during treatment, were observed for 4 h. As shown in the figure 2, change in the temperature of the inner flask space was gradual and it took about 90 min to reach to the equilibrium with the TIC.

After transferring the flask back to the growth room, temperature of the inner flask space gradually decreased and again it took about 90 min to reach to equilibrium with the growth room

temperature. During 3 h treatment, plantlets experienced 90 min gradual increasing of temperature and subsequently 90 min of constant TST after reaching to temperature equilibrium, and finally 90 min of gradual decreasing temperature during transferring the flasks back to the growth room.



Figure 1. Shoot cultures of *L. persicum* used for the thermal-stress treatment (TST).

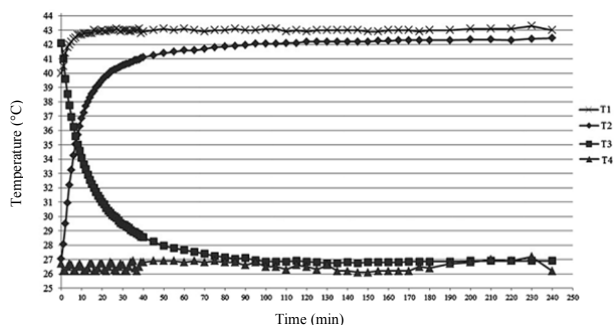


Figure 2. Temperature profiles in the thermal-stress evaluation of *L. persicum* shoot cultures. **T**₁, thermally isolated cabin (TIC) adjusted at 43 °C; **T**₂, inner of the flasks after transferring from growth room to the TIC; **T**₃, inner of the flasks after transferring back from TIC to the growth room; **T**₄, growth room.

The heat transfer condition in culture flasks included conduction, convection, and radiation, which depended on flasks physical properties [16]. The low conduction properties of the glasses, the green house effects inside the flasks, and the low energy transfer by convection due to nearly closed system of the culture vessels may explain these observations. After transferring back the flasks from TIC to the growth room,

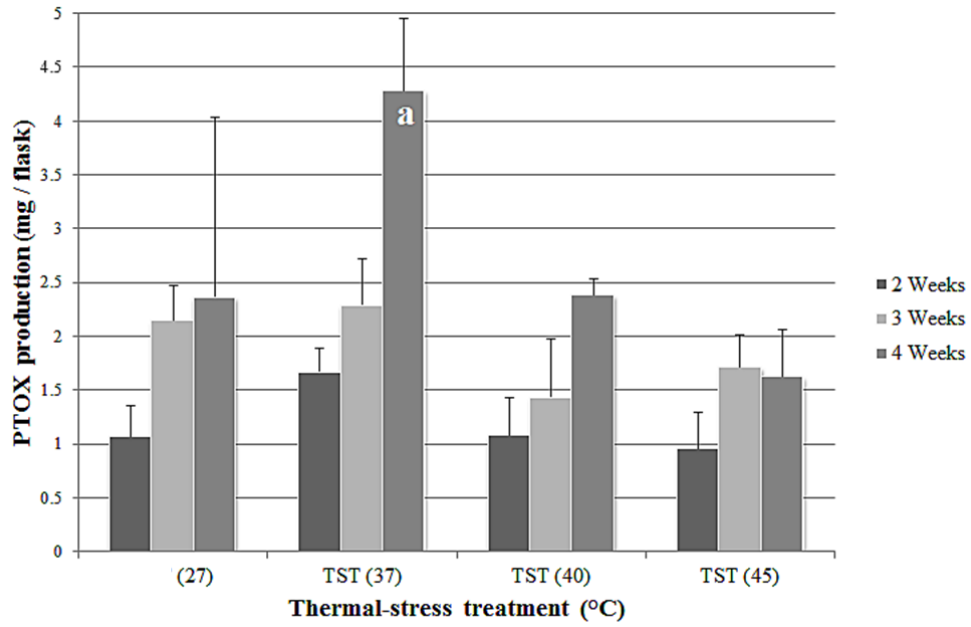


Figure 3. The PTOX productivity (mg/flask) of *L. persicum* shoot cultures under thermal-stress. **TST:** thermal-stress treatment at the corresponding temperature. ^a Significant differences to all other groups, n=4, p< 0.05.

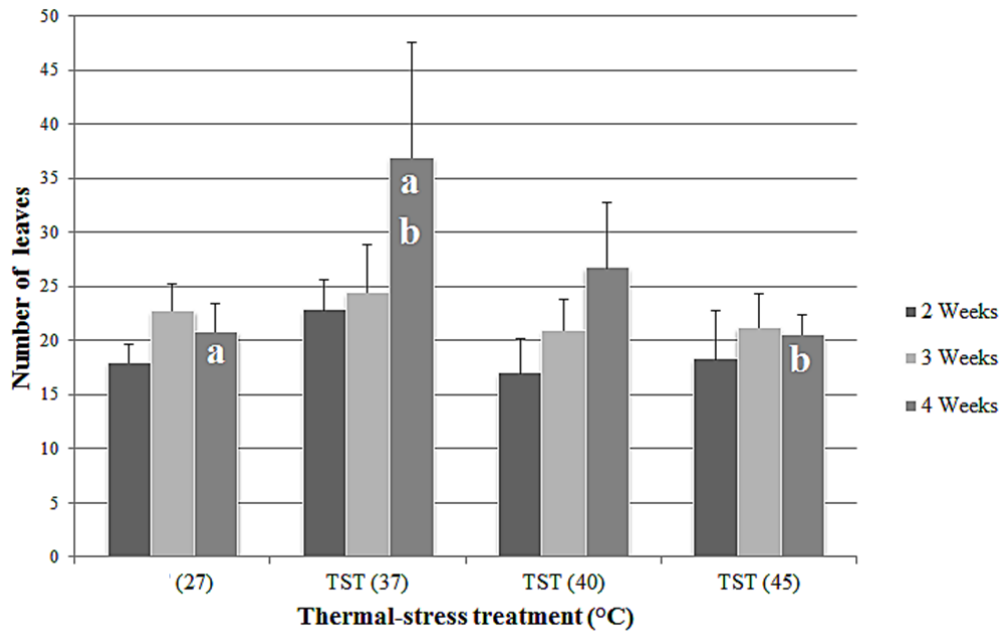


Figure 4. Effect of thermal-stress on the number of leaves of *L. persicum* shoot cultures. **TST:** thermal-stress treatment at the corresponding temperature. ^{a, b} Significant differences to corresponding a or b, n=4, p< 0.05.

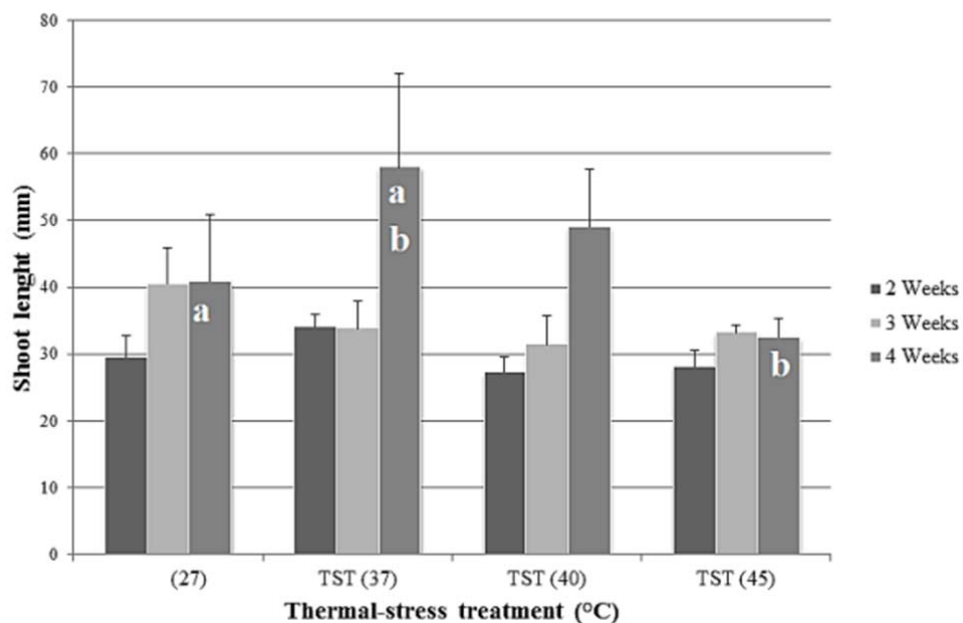


Figure 5. Effect of thermal-stress on shoot length (mm) of *L. persicum* shoot cultures. **TST:** thermal-stress treatment at the corresponding temperature. ^{a, b} Significant differences corresponding a or b, n=4, $p \leq 0.05$.

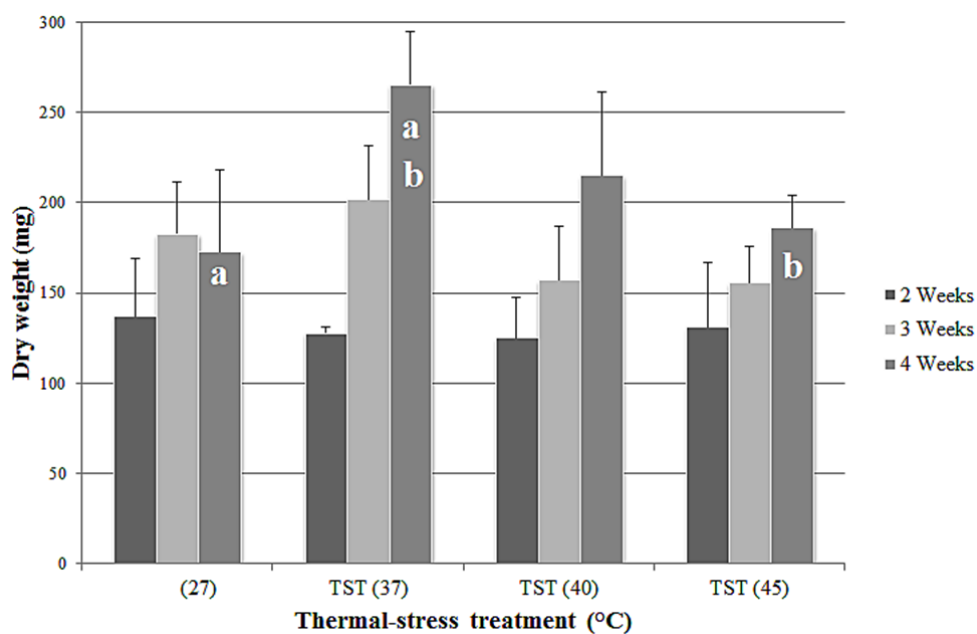


Figure 6. Effect of thermal-stress on dry weight (mg) of *L. persicum* shoot cultures. **TST:** thermal-stress treatment at the corresponding temperature. ^{a, b} Significant differences corresponding a or b, n=4, $p \leq 0.05$.

samples which were treated at 50 and 55 °C started browning and after 2 days they were completely dead. Thus samples in these 2 groups were not used for PTOX analysis and shoot proliferation measurements.

The effect of TST on PTOX production per flask is shown in figure 3. Samples which were heat-stressed at 37 °C, and extracted four weeks after the treatment, showed the highest amount of PTOX productivity per flask compared to all other groups ($p \leq 0.05$). Bowen *et al.* [17] reported that the elevation of temperature would result in heat shock response which is characterized by rapid induction of heat shock protein gene transcription simultaneously with a decline in the transcription of other genes. As described by Federolf *et al.* [18] the biosynthesis of PTOX starts with the general phenylpropanoid pathway. The principal enzyme of this pathway is phenylalanine ammonialyase (PAL).

The accumulation of PTOX was up to 1.62 ± 0.21 %DW. Samples which had experienced 37 °C of TST, possessed significantly ($p \leq 0.05$) higher number of leaves (figure 4) and shoot length (figure 5) 4 weeks after the treatment compared to other groups.

One of the main acclimatory responses of cells to TST is the increased activity of PAL [13], therefore the observed increase in PTOX production in this study might be due to the increase in the activity of PAL. It seems that increasing in culture temperature caused increase in horizontal growth of shoots to some extent, but further increasing in temperature caused reduction of horizontal growth. The same effects were observed in the leaves proliferation and significantly ($p \leq 0.05$) higher DW was observed for 37 °C compared to 40 °C and 45 °C (figure 6). According to the increase in DW simultaneously with increase in PTOX production, it could be concluded that optimized TST could result in increase of PTOX productivity to 80%.

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