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Original Article

Potential of Podophyllotoxin production in some Iranian Ecotypes of *Linum album* under *in vitro* Condition

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ABSTRACT

Linum album Kotschy ex Boiss. as one of the endemic perennial plants in Iran is a natural source of lignan compounds. In the present study, the seeds of *L. album* were collected from its natural habitats in four regions of Iran (Aliabad, Jowkar, Dasht-e Arzhan, Taleqan). The effects of ecotype and gibberellic acid (GA) pretreatment were evaluated on germination traits of *L. album* seeds in a factorial based on completely randomized design with four replications. Also, the capacity of studied ecotypes in regard to secoisolariciresinol (SECO), podophyllotoxin (PTOX) and 6-methoxy-podophyllotoxin (6-MPTOX) production was assayed under *in vitro* condition by high performance liquid chromatography (HPLC) based on completely randomized design with four replications. In all studied ecotypes of *L. album*, seed germination percentage, germination rate and seedling growth was significantly improved in response to GA pretreatment. The obtained sterilized seedlings were used as *in vitro* explants that were successfully propagated in MS medium without any growth regulators. The *in vitro* proliferation rate in ecotype of Jowkar in terms of shoot number and plantlet biomass was significantly higher than the other ecotypes. The highest SECO and PTOX contents were extracted in the Dasht-e Arzhan plantlets which were higher (up to four times) than the other ecotypes. Also, the plantlets of Dasht-e Arzhan, Taleqan and Jowkar ecotypes showed the highest content of 6-MPTOX. According to germination, morphological and phytochemicals traits, the ecotype of Dasht-e Arzhan can be considered as a good candidate for breeding programs of improving PTOX production in this plant.

INTRODUCTION

Secondary metabolites are synthesized in a wide variety in different plant species and play a key role in the interaction of plants with environment challenges. Also due to biological activity of these compounds, secondary metabolites have been used to enhance different aspects of human health, diet and welfare [1]. Unlike primary compounds, the secondary metabolites are usually restricted to particular plant families, genera or even species as well as the synthesis and accumulation of these compounds may only occur at a specific plant development stage in special organs or tissues [2]. The *in vitro* culture of different plant organs or tissues (embryo, shoot, leaf, root, etc.) has been used in production of secondary metabolites in different plant species as an alternative approach, especially in species with low growth rate or low abundance in the wild. In the method of organ culture, secondary products are synthesized in similar patterns of a mature plant [3, 4]. Although the tissue and organ culture is an alternative procedure for the production of different types of secondary metabolites but to date the commercialization of this method is still a major concern. This problem is due to the low concentration of produced secondary metabolites under *in vitro* culture systems [5,6].

The lignans as a group of chemical compounds found in plants are derived-polyphenolic

compounds through dimerization of substituted cinnamic alcohols in phenylalanine metabolism [7]. The podophyllotoxin (PTOX) as natural aryltetralin lignan is a species dependent compound that is synthesized through specific plant secondary metabolism. PTOX is the main raw material and the precursor for the production of anticancer drugs to treat different human cancers [8, 9]. The lignan of PTOX and the other related compounds such as 6-methoxy-podophyllotoxin (6-MPTOX) and secoisolariciresinol (SECO) are synthesized through the pathways of shikimic acid and phenylpropanoid with involving of different enzymes and intermediates [10]. The matairesinol is reported as a common intermediate in the podophyllotoxin production pathway [1]. However, the related sub-pathways in PTOX synthesis have not been completely understood and may vary among the different plant species [10].

For many years, the rhizome of *Phodophyllum* spp. especially *P. hexandrum* has been the major resource for PTOX production. However, excessive and incorrect harvesting of this plant and low efficiency of solvent extraction method have limited the production of PTOX from *P. hexandrum* [6]. Also the ambiguous points in the podophyllotoxin production pathways make its chemical synthesis uneconomical under laboratory condition [9]. Nowadays applied biotechnological means are attracted the attention of several researchers for PTOX production [5]. Bahabadi *et al*. [11] suggested that biotechnological methods can be a viable alternative way for the production of PTOX and the related products in *Linum album*. Yousefzadi *et al*. [12] assayed the potential of more than 40 plants and fungal species for PTOX production under *in vitro* conditions. These authors reported that among the studied species, the extracted PTOX from *in vitro* plantlets of *Podophyllum* spp., *Callistris drummondii* and the Iranian *Linum* species (*L. album* and *L. persicum*) were in significant amounts. Also, the results of Ahmadian Chashmi *et al*. [7]; Bose *et al*. [13]; Esfandiari *et al*. [14] and Mikac *et al*. [15] confirmed that the *Linum* spp. including *L. album* are valuable and alternative sources of podophyllotoxin and other important lignans. It seems that developing a large-scale culture of *L. album* under *in vitro* condition can provide the required PTOX of different medicine industries.

It should be noted that the content of secondary metabolites is influenced by genetic factors and may vary between plant species even within species. Environmental conditions have also considerable effects on quality and quantity of active substances in medicinal plants [16]. So that in the present study the seeds of *L. album* (white flax) were collected from four different regions of Iran and their germination traits were tested. Also, under *in vitro* condition, the production capacity of these ecotypes in regards to PTOX, SECO and 6-MPTOX was investigated. Finally, the superior ecotype was selected based on germination traits, morphological and phytochemicals characteristics of *in vitro* proliferated plantlets.

MATERIALS AND METHOD

Plant Material

The seeds of *L. album* were collected from natural habitats in four regions of Iran including Aliabad (Hamadan province), Jowkar (Hamadan province), Dasht-e Arzhan (Fars province) and Taleqan (Alborz province). The geographical coordinates of the collected sites are presented in the Table 1. Identification of ecotypes was conducted by Dr. Vahideh Nazeri Jounghani, and voucher specimens (voucher number: 6491) have been deposited at the Herbarium of Faculty of Agricultural Sciences and Engineering, University of Tehran.

Table 1. Geographical origins of the of *Linum album* Kotschy ex Boiss.ecotypes from different part of Iran *Linum album* Kotschy ex Boiss. ecotypes.

Ecotype No	Collecting place	Province	Altitude (m)	Latitude (N)	Longitude (E)
1	Dasht-e Arzhan	Fars	1990	29° 39′	51° 59′
2	Aliabad	Hamadan	2134	34° 41′	48° 38′
3	Jowkar	Hamadan	1751	34° 22′	48° 40′
4	Taleqan	Alborz	1938	36° 09′	50° 41′

Assay of Breaking Seed Dormancy in Different Ecotypes of *L. album*

The 1000 seed weight of each collected ecotype was measured and due to endogenous and exogenous dormancy of *L. album* (white flax) seeds, the first experiment was performed to assay the effect of gibberellic acid (GA) pretreatments to break dormancy of white flax seeds. For this purpose, after washing with running tap water for 24 h, the seeds were treated with distillated water as control and GA (1000 ppm) for 24 h at 4 °C. The mucilage of seeds' surface was removed by thin fabric. After that the seeds were sterilized with 5% sodium hypochlorite for 15 min and then rinsed three times with distilled water. The pretreated seeds (50 seeds) were placed in sterile petri dishes containing filter paper and then kept in a growth chamber at 25 ± 1 °C. Root emergence (at least one millimeter long) was considered as germinated. Germinated seeds were counted daily so that the counting was done continuously until the end of germination (after 21 days). At the end of experiment, the germination percentage, germination rate, seedling length, root length, as well as fresh and dry weight of seedlings were measured. The effect of ecotype and GA treatment on germination traits of *L. album* seeds was performed in a factorial based on completely randomized design with four replications and 50 seeds per replicate.

Proliferation of *L. album* under *in vitro* Culture

For disinfection and production of sterile seedlings, after washing with running tap water for 24 h, the seeds were sterilized with 70% ethanol for one min and then 5% sodium hypochlorite for 10 minutes. Although by this method the fungal symptoms were removed but the bacterial contamination was still observed in the seeds. To control the bacterial contamination, the seeds were washed with an antibiotic, vancomycin (20 ppm) for five minutes and then rinsed with distilled water. Finally, the seeds were soaked in milton sodium dichloroisocyanurate solution for 24 h. Using this method, the fungal and bacterial contamination were well controlled. According to the results of the germination experiment as reported in previous part, pretreatment by GA (1000 ppm) was applied for improving seed germination. Then the seeds were cultured in MS medium [17] and were placed in a dark place at 20 °C [18]. After three days, more than 50% of seeds showed germination. The obtained sterilized 8-10 day – old *L. album* seedlings were used as explants for *in vitro* propagation. Shoot explants (10–12 mm long) were excised from the seedlings and transferred to MS medium. All the cultures maintained at 25±1 °C under 16/8 h photoperiod. For further proliferation, the sub-culturing was carried out on the same media every two weeks for three months. In the obtained plantlets, the morphological and phytochemical characters were assayed.

The cultured media contained MS mineral, vitamins, sucrose (3% w/v) and solidified with agar (0.8% w/v) [17]. The pH of the media was adjusted to 5.7 and then autoclaved for 20 min at 121 °C. The studied morphological traits included the number of shoots per plantlet, the length of the highest shoot, fresh and dry weight of plantlets. The assay of ecotype effect on morphological traits of proliferated *L. album* plantlets was carried out on the base of completely randomized design with four replications and six samples per each replicate.

Assay of Phytochemical Traits in the Propagated Plantlets

The lignan compounds including secoisolariciresinol (SECO), podophyllotoxin (PTOX) and 6-methoxy podophytotin (6-MPTOX) were measured in *in vitro* proliferated plantlets. For this purpose, 1 g dry tissue from the pooled mixture of six samples was homogenized with 10 mL 80% methanol. The extraction was placed on ultrasonic apparatus (Parsonic 7500S) for 90 min. Then equal amounts of water and dichloromethane (10 mL) were added to the obtained solution and shaken for 15 min. The mixture was then centrifuged at 4000 rpm at 10 °C for 20 minutes. After centrifugation, the lower phase (dichloromethane phase) was collected and dried by aeration. To the dried sample, 2 mL of HPLC grade methanol was added and centrifuged at 1000 rpm for 10 min. The supernatant was used for quantification of SECO, PTOX and 6-MPTOX. The content of the lignan compounds was measured by the high-performance liquid chromatography (HPLC Waters 2695 USA). A Waters liquid chromatography apparatus consisting of a separation module and a PDA detector (Waters 996 USA) was used for the HPLC analysis. Data acquisition and integration was performed with Millennium 32 software. Injection was carried out by the auto sampler injector. The chromatographic

assay was performed on a 25 cm × 4.6 mm with pre-column, Eurospher 100-5 C18 analytical column provided by KNAUER reverse phase matrix (5 µm) and elution was carried out in a gradient system with acetonitrile as the organic phase (solvent A:70%) and distilled water (solvent B: 30%) with the flow-rate of 1 ml/min. Peaks were monitored at 280 nm wavelength. Injection volume was 20 µL and the temperature was maintained at 25 °C [12]. The content of lignan compounds was calculated according to standard curves of SECO, PTOX and 6-MPTOX. For this purpose, different concentrations of SECO, PTOX and 6-MPTOX at 10, 50, 100 and 200 ppm were prepared and mixed with each other. Then the obtained solution was injected to HPLC and the content of SECO, PTOX and 6-MPTOX used for drawing the standard curves of each compound. The results were reported as mg of each compound per g of plantlet dry weight. The assay of ecotype effect on lignan compounds of proliferated *L. album* plantlets was carried out on the base of completely randomized design with four replications and six samples per each replicate

Statistical Analysis

All data were analyzed using SPSS software (ver.16.0). LSD tests at $p \leq 0.05$ were used to comparison mean values of each trait between different ecotypes.

RESULTS

The analysis of the means difference for 1000-seed weight was significant. The 1000-seed weight of collected seeds from Taleqan (3385 mg) and Dasht-e Arzhan (3262.5 mg) was significantly higher than Aliabad (2812.5 mg) and Jowkar (2761.75 mg) ecotypes (Fig. 1).

The results of the analysis of variance showed a significant effect of GA pretreatment on seed germination. The white flax seeds begun to sprout 24 h after culturing. In all four studied ecotypes, GA considerably improved germination percentage and germination rate of treated seeds. In the GA treatment, the highest germination percentage was observed in Taleqan ecotypes (94.37%) followed by Dasht-e Arzhan (87.18%) and Jowkar (86.87%) ecotype. The seeds of Aliabad with 77.07% showed the lowest germination percentage (Table 2). The seeds of Aliabad ecotypes despite showing the lowest germination percentage, had the higher germination rate (0.435 day) compared to other studied ecotypes. The seeds of Taleqan ecotype with the highest germination percentage showed the lowest germination rate (Table 2).

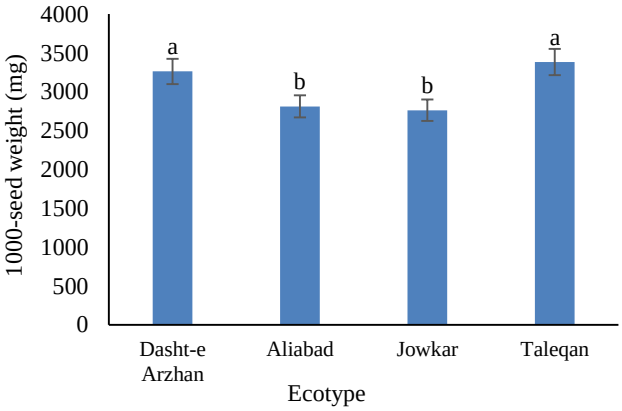

Fig. 1 The 1000 seed weight of different *Linum album* Kotschy ex Boiss. Ecotypes**.** Different letters indicate significant difference by Duncan multiple range test at $p \leq 0.05$.

The growth of obtained seedlings in regard to height, fresh and dry weight as well as root growth was significantly influenced by GA pretreatment. GA treatment considerably improved the seedling and root growth. In the treated seeds, the highest seedling height (191.58 mm) and root length (122.58 mm) were observed in Dasht-e Arzhan ecotype, which were not significantly different from seedlings of Jowkar ecotype. In the GA treatment, the fresh and dry weight of obtained seedlings of Dasht-e Arzhan, Jowkar and Taleqan ecotypes did not show a significant difference. The biomass of Aliabad ecotype was considerably lower than the other studied ecotypes (Table 2).

According to the results of the first experiment, for improving seed germination of studied ecotypes GA was used for seed pretreatment in the next *in vitro* experiments. The GA pretreated seeds were successfully germinated in MS medium and the obtained seedlings were then proliferated under *in vitro* condition (Fig. 2). Analysis of variance showed the non-significant differences on four studied *L. album* ecotypes in regard to shoot height. The data analysis of shoot number and plantlets biomass revealed significant differences among the ecotypes. The highest shoot number, fresh and dry weight of plantlets were observed in ecotype of Jowkar. The dry weight of propagated plantlets of Dasht-e Arzhan and Aliabad had no significant difference with those of Jowkar ecotype.

Table 2 The effect of gibberellic acid pretreatment on seed germination traits of different *Linum album* Kotschy ex Boiss. ecotypes

Gibberellic acid (ppm)	Ecotype	Germination percentage (%)	Germination rate (day)	Seedling length (mm)	Root length (mm)	Seedling fresh weight (mg)	Seedling dry weight (mg)
0	Dasht-e Arzhan	57.50 cd	0.215 d	131.85 bc	85.82 bc	490.80 b	49.97 bc
	Aliabad	53.15 d	0.163 e	106.14 cde	69.35 bc	401.10 bc	59.05 ab
	Jowkar	52.88 d	0.233 d	65.25 e	24.87 d	333.95 c	35.85 c
	Taleqan	65.63 c	0.208 d	86.25 de	65.55 bc	328.75 c	39.95 c
1000	Dasht-e Arzhan	87.18 ab	0.333 b	191.58 a	122.58 a	668.25 a	62.42 ab
	Aliabad	77.07 b	0.435 a	111.18 cd	54.30 cd	423.00 bc	39.82 c
	Jowkar	86.87 ab	0.335 b	162.65 ab	99.25 ab	665.75 a	65.95 a
	Taleqan	94.37 a	0.280 c	135.53 bc	77.08 bc	627.32 a	56.27 ab

Values followed by the same letter within a column indicate not significantly difference ($p< 0.05$).

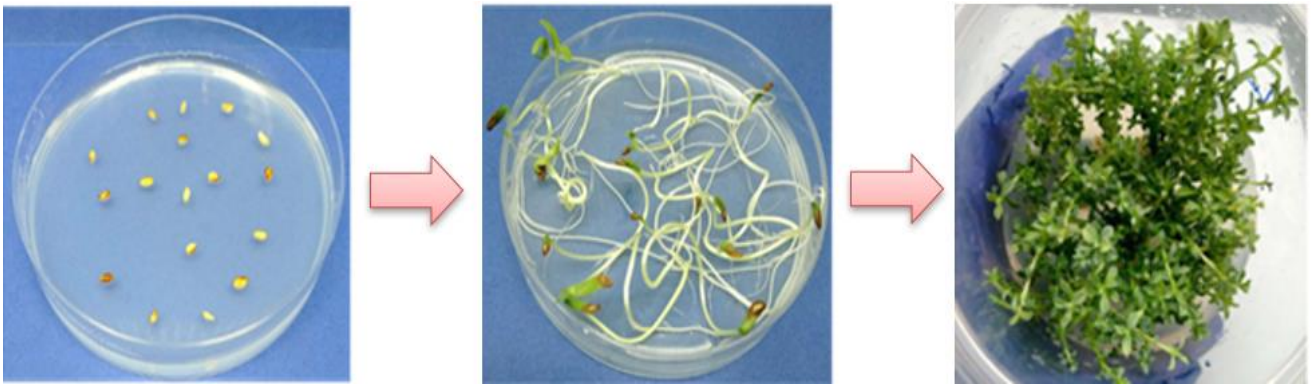

Fig. 2 The production of sterilized seedling of *L. album* under *in vitro* condition (Dasht-e Arzhan ecotype)

Table 3 The morphological traits of propagated plantlets of different *Linum album* Kotschy ex Boiss. ecotypes under in vitro condition

Ecotype	Shoot length (cm)	Shoot number (per plantlet)	Plantlet fresh weight (g)	Plantlet dry weight (g)
Dasht-e Arzhan	14.25 a	22.70 b	2.99 b	0.34 ab
Aliabad	11.54 a	16.75 b	3.03 b	0.36 ab
Jowkar	12.92 a	40.50 a	5.16 a	0.45 a
Taleqan	11.92 a	13.80 b	2.02 b	0.26 b

Values followed by the same letter within a column indicate not significantly difference ($p< 0.05$).

Table 4 The phytochemical content of propagated plantlets of different *Linum album* Kotschy ex Boiss. ecotypes under in vitro condition

Ecotype	SECO (mg/g DW)	PTOX (mg/g DW)	6-MPTOX (mg/g DW)
Dasht-e Arzhan	0.045 a	0.057 a	0.100 a
Aliabad	0.015 b	0.012 b	0.005 b
Jowkar	0.018 b	0.031 b	0.046 ab
Taleqan	0.011 b	0.022 b	0.094 a

Values followed by the same letter within a column indicate not significantly difference ($p< 0.05$).
DW: dry weight; SECO: Secoisolariciresinol; PTOX: Podophyllotoxin; 6-MPTOX: 6-methoxy-podophyllotoxin

The results showed that the Taleqan ecotype had the lowest efficiency in terms of the studied traits under *in vitro* condition (Table 3).

The phytochemical compounds assay of *in vitro* plantlets of *L. album* showed significant differences among the four studied ecotypes. The highest amount of SECO (0.045 mg/g DW) was found in

the Dasht-e Arzhan plantlets, which was up to four times higher than the other ecotypes. The SECO content in the other three ecotypes had not significant differences with each other (Table 4). The plantlets of Dasht-e Arzhan also showed the highest PTOX content 1.84, 2.59 and 4.75 times more than its content in Jowkar, Taleqan and Aliabad ecotypes, respectively (Table 4). According to the reported results in Table 4, the highest content of 6-MPTOX was obtained in the plantlet of Dasht-e Arzhan (0.1 mg g^{-1} DW) and Taleqan (0.094 mg g^{-1} DW) ecotypes followed by Jowkar ecotype (0.046 mg g^{-1} DW). The plantlets of Aliabad ecotype showed the lowest content of 6-MPTOX which considerably was lower than the other ecotypes (Table 4). The HPLC chromatogram of lignans analysis in studied *L. album* ecotypes was depicted in Fig 3.

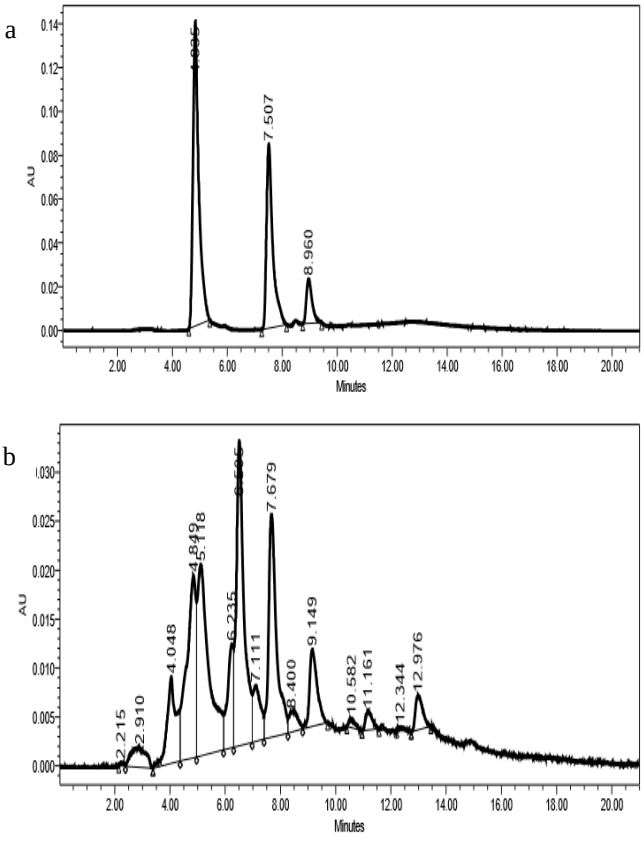

Fig. 3 The HPLC chromatogram of lignans analysis in a: standard and b: Dasht-e Arzhan ecotype of *L. album*

DISCUSSION

The obtained results revealed the considerable variations in the 1000-seed weight among the studied *L. album* ecotypes. The results of Kiani [18], and Kiani *et al*. [19] also demonstrated the significant differences in the seed traits of Iranian *L. album* ecotypes. The differences in the seed characteristics of different ecotypes were attributed to this notice that flowering and seed filling processes are affected by genetic factors, morphological, physiological and ecological characteristics as well as their interaction [20].

Ashrafi *et al*. [21] reported the very slow germination of *L. album* seeds due to exogenous and endogenous dormancy. In the present study the seed pretreatment of *L. album* ecotypes using gibberellic acid considerably improved seed germination, seed rate and seedling growth. The effect of GA on seed breaking dormancy and improving germination of different species of *Linum* has been previously reported by Samadi *et al*. [22]. Many physiological and molecular mechanisms regulate seed dormancy. The accumulation of growth inhibitors especially abscisic acid (ABA) causes the embryo dormancy of seeds; however, the dormancy breaking is associated to increasing the growth promoter compounds that reduces the effect of growth inhibitors [23]. The enchantment of seed germination in GA treatment is attributed to eliminating the chilling requirement of treated seeds [24]. In addition to endogenous dormancy, the presence of mucilage on the seed coat of *L. album* causes a physical barrier which rinsing seeds by water eliminated this barrier. In the studied ecotypes, the highest germination percentage and germination rate were obtained in Taleqan and Aliabad ecotypes, respectively. The growth of seedlings in ecotype from Dasht-e Arzhan in regards to height and biomass were more prominent than the other studied ecotypes (Table 2). Similar results were found by Ashrafi *et al*. [21] in different species of *Linum* genus. Despite these results, Mahdavi and Alasvandyari [25] reported no significant differences in seed germination traits among the *L. usitatissimum* ecotypes. The variation in seed dormancy and germination traits of different ecotypes may reflect the differences in climatic conditions of their habitats [23,24]. It is reported that the environmental conditions during seed maturation may have a greater effect than genetic factors in the seed dormancy level and germination potential [26].

Under *in vitro* condition, up to 50% of cultured seeds in all studied ecotypes of *L. album* were started to germinate after three days and the germination of seeds was completed after 8-10 days. The obtained seedlings (Fig. 2) were used as *in vitro*

explants that were successfully propagated in MS medium without any growth regulator. According to Lalaleo *et al*. [6] reports the best condition for growth and organogenesis of *L. album* was free plant growth regulators medium either in light or in darkness conditions. Increasing the secondary metabolites without reduction of the biomass is the main point in optimizing production of plant active substances through biotechnological process [1]. The plantlets of studied ecotypes did not show differences in the shoot height. The *in vitro* proliferation rate of Jowkar ecotype in terms of shoot number and plantlet biomass was significantly higher than the other ecotypes. However, the plantlet dry weight in the ecotypes of Dasht-e Arzhan and Aliabad did not show statistically any differences with Jowkar ecotype (Table 3). The differences in genotypes and geographical conditions are responsible for the variation in response of different ecotypes under *in vitro* culture [3, 22].

Nowadays due to climatic changes and plant challenging with environmental conditions, *in vitro* culture is the interest of many researchers for the sustainable production of secondary metabolites [15]. The low yield of podophyllotoxin in the commercial source of this compound from *Podophyllum hexandrum* rhizomes (up to 5% of dry weight) caused the introduction of alternative natural sources of podophyllotoxin [6]. In the last decades, several alternative sources of podophyllotoxin such as Linaceae, Cupressaceae, Lamiaceae, Polygalaceae and Podophyllaceae have been identified [9, 27]. Assay their capacities for lignan accumulation showed that some *Linum* species can be promising sources of PTOX [28]. Also, Baldi *et al*. [29] reported that the production of podophyllotoxin through *in vitro* culture of *Linum* spp. had a higher potential in comparison to *in vitro* cultures of other plants. The *L. album* as Iranian endemic plant is not extensively cultivated so that *in vitro* cell or tissue culture can be favored over the whole plant culture for the production of lignan metabolites [5]. However, the main step in optimizing the biotechnological production of secondary metabolites is the evaluation and selection of the elite plant lines with high quantity and quality of these compounds [4]. The production of lignan compounds especially PTOX from *in vitro* culture of *L. album* were investigated by several researchers [5, 7, 30] but most of these literatures focused on cell cultures of *L. album*. The results of Empt *et al*. [30] as the first published paper on lignans production in cell culture of *L. album*, reported the accumulation of PTOX up to 0.5 % in dry weight of samples. In the present study, significant differences were found in the SECO, PTOX and 6-MPTOX content in the *in vitro* plantlets of *L. album* ecotypes. The highest SECO and PTOX contents were obtained in the Dasht-e Arzhan plantlets being up to 4 times higher than the other ecotypes. Also, the plantlets of Dasht-e Arzhan, Taleqan and Jowkar ecotypes showed the highest content of 6-MPTOX. Among the studied ecotypes, the *in vitro* produced plantlets of Aliabad had the lowest potential in regard to the studied lignans in this study (Table 4). Differences in phytochemical contents within a species found in various ecotypes may reflect the differences in environmental conditions of each particular region [26, 31]. The PTOX content in Dasht-e Arzhan plantlets (0.057 mg g^{-1} dry weight) was higher than the findings of Esmaeilzadeh Bahabadi *et al*. [32]; Sedaghat *et al*. [33] who reported 18 and 5 µg PTOX per g dry weight in cell cultures of *L. album.* However, discrepancy among the results of literatures may be related to differences in genotypes [31], climatic and edaphic conditions of collecting area [15] type of explant [4], culture media [12], extraction methods and assay apparatus [12].

CONCLUSION

In the present study the seed germination, morphological traits and lignans production of four Iranian *L. album* ecotypes were evaluated under *in vitro* condition. Generally, the obtained results showed that GA pretreatment was efficient in enhancement of seed germination and seedling growth of *L. album* ecotypes. Also, the studied ecotypes were successfully proliferated under *in vitro* condition. The assay of SECO, PTOX and 6-MPTOX contents indicated a significant variation of these compounds among the four Iranian *L. album* ecotypes. The plantlets of Dasht-e Arzhan that were collected from Fars province showed the highest content of the lignans compounds. With consideration of highest growth and the lignan content in the Dasht-e Arzhan ecotype, this ecotype

can be suggested for further breeding programs as an alternative source of PTOX production.

Declarations

Authors Contribution

Manizhe Abdollahpoor: Experimental work, data analysis, writing.

Vahide Nazeri: Experimental design, supervision, writing.

Majid Shokrpour: Experimental design, data analysis.

Ardeshir Qaderi: Experimental work, supervision.

Khalil-Berdi Fotouhifar: Experimental design, supervision.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Satake H., Koyama T., Bahabadi S.E., Matsumoto E., Ono E., Murata J. Essences in metabolic engineering of lignan biosynthesis. Metabolites. 2015;4:70-90. doi: 10.3390/metabo5020270. PMID: 25946459; PMCID: PMC4495373.
2. Thirumurugan D., Cholarajan A., Vijayakumar SSRA. An introductory chapter: secondary metabolites. In: Vijayakumar R, Raja SS. (Eds.), Secondary metabolites - sources and applications. IntechOpen. 2018. https://doi.org/10.5772/intechopen.79766
3. Chandran H., Meena M., Barupal T., Sharma K. Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. Biotechnol Rep (Amst). 2020;26:e00450. doi: 10.1016/j.btre.2020.e00450
4. Espinosa-Leal C.A., Puente-Garza C.A., García-Lara S. *In vitro* plant tissue culture: means for production of biological active compounds. Planta. 2018;248:1-18. https://doi.org/10.1007/s00425-018-2910-1
5. Javadian N., Karimzadeh G., Sharifi M., Moieni A., Behmanesh M. *In vitro* polyploidy induction: changes in morphology, podophyllotoxin biosynthesis, and expression of the related genes in *Linum album* (Linaceae). Planta. 2017;245:1165-1178.
6. Lalaleo L., Testillano P., Risueño M.C., Cusidó R.M., Palazon J., Alcazar R., Bonfill M. Effect of *in vitro* morphogenesis on the production of podophyllotoxin derivatives in callus cultures of *Linum album*. J Plant Physiol. 2018;228:47-58. doi: 10.1016/j.jplph.2018.05.007.
7. Ahmadian Chashmi N., Sharifi M., Behmanesh M. Lignan enhancement in hairy root cultures of *Linum album* using coniferaldehyde and methylenedioxycinnamic acid. Prep Biochem Biotechnol. 2016;46:454-460. doi: 10.1080/10826068.2015.1068802. PMID: 26444150.
8. Lalaeo L, Khojasteh A, Fattahi M, Bonfill M, Cusido RM, Palazon J. Plant anti-cancer agents and their biotechnological production in plant cell biofactories. Curr Med Chem. 2016;23:4418-4441.
9. Shah Z, Gohar UF, Jamshed I., Mushtaq A., Mukhtar H., Zia-UI-Haq M., Toma S.I., Manea R., Moga M., Popovici B. Podophyllotoxin: History, recent advances and future prospects. Biomolecules. 2021;11:603. https://doi.org/10.3390/biom11040603
10. Kumari A., Dogra V., Joshi R., Kumar S. Stress-responsive *cis*-regulatory elements underline podophyllotoxin biosynthesis and better performance of *Sinopodophyllum hexandrum* under water deficit conditions. Front Plant Sci. 2022;4:751846. doi: 10.3389/fpls.2021.751846. PMID: 35058943; PMCID: PMC8764236.
11. Bahabadi S.E., Sharifi M., Chashmi N.A., Murata J., Satake H. Significant enhancement of lignan accumulation in hairy root cultures of *Linum album* using biotic elicitors. Acta Physiol Plant. 2014;36:3325-3331.
12. Yousefzadi M., Sharifi M., Behmanesh M., Moyano E., Bonfill M., Cusido R.M., Palazon J. Podophyllotoxin: Current approaches to its biotechnological production and future challenges. Eng in Life Sci. 2010;10:281-292.
13. Bose S., Munsch T., Lanoue A., Garros L., Tungmunnithum D., Messaili S., Destandau E., Billet K., St-Pierre B., Clastre M, Abbasi B.H., Hano C., Giglioli-Guivarc'h N. UPLC-HRMS analysis revealed the differential accumulation of antioxidant and anti-aging lignans and neolignans in *in vitro* cultures of *Linum usitatissimum* L. Front Plant Sci. 2020;11:508658. doi: 10.3389/fpls.2020.508658. PMID: 33072140; PMCID: PMC7539065.
14. Esfandiari M., Sharifi M., Mohamadyar-Toupkanlou F., Hanaee-Ahwaz H., Yousefzadi M., Jafari A., Hosseinzadeh S., Soleimani M. Optimization of cell/tissue culture of *Linum persicum* for production of lignans derivatives including Podophyllotoxin. Tissue Organ Cult. 12018;33:51-61.
15. Mikac S., Markulin L., Drouet S., Corbin C., Tungmunnithum D., Kiani R., Kabra A., Abassi B.H., Renouard S., Fuss E. Bioproduction of Anticancer Podophyllotoxin and Related Aryltretralin-Lignans in Hairy Root Cultures of *Linum flavum* L. In: Ramawat K, Ekiert H, Goyal S (eds.) Plant Cell and Tissue Differentiation and Secondary Metabolites, Springer, Cham, Switzerland. 2020, pp. 1-38.
16. Zare S., Mirlohi A., Saeidi G., Sabzalian M.R., Ataii E. Water stress intensified the relation of seed color with lignan content and seed yield components in flax (*Linum usitatissimum* L.). Sci Rep. 2021;11:23958. https://doi.org/10.1038/s41598-021-02604-5.

17. Murashige T., Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant. 1962;15:473-497
18. Kiani R. Evaluation of morphological and phytochemical diversity of *Linum album* Ky. ex Bioss., an endemic medicinal plant in Iran. Master's Thesis, Department of Horticultural Sciences College of Agriculture and Natural Resources, University of Tehran, Tehran, Iran. 2016.
19. Kiani R., Nazeri V., Shokrpour M., Hano C. Morphological, physiological, and biochemical impacts of different levels of long-term water deficit stress on *Linum album* Ky. ex Boiss. accessions. Agro. 2020;10:1966. https://doi.org/10.3390/agronomy10121966
20. Sehgal A., Sita K., Siddique KH.M., Kumar R., Bhogireddy S, Varshney RK, HanumanthaRao B, Nair RM, Prasad PVV, Nayyar H. Drought or/and heat-stress effects on seed filling in food crops: Impacts on functional biochemistry, seed yields, and nutritional quality. Front Plant Sci. 2018;9:1705. doi: 10.3389/fpls.2018.01705. PMID: 30542357; PMCID: PMC6277783
21. Ashrafi E., Nadali M., Taheri S. Breaking seed dormancy of *Linum album*, a medicinal plant of the Iran. Bio. 2013;85655644
22. Samadi A., Carapetian J., Qadim Zadeh M., Hassanzadeh Ghortapeh A. Comparison of two culture media for breaking seed dormancy and germination improvement in four species of *Linum* L. Afr J Biotechnol. 2012;11:4699-4705.
23. Matilla A.J. Seed dormancy: Molecular control of its induction and alleviation. Plants. 2020;9:1402.
24. Klupczynska EA, Pawłowski TA. Regulation of seed dormancy and germination mechanisms in a changing environment. Int J Mol Sci.2021; 22:1357. https://doi.org/10.3390/ijms22031357
25. Mahdavi B., Alasvandyari F. Germination and morphophysiological responses of flax (*Linum usitatissimum* L.) ecotypes to salinity stress. Journal of Plant Physiology and Breeding. 2019;8:77-87.
26. Carta A., Hanson S., Müller J.V. Plant regeneration from seeds responds to phylogenetic relatedness and local adaptation in mediterranean *Romulea* (Iridaceae) species. Ecol Evol. 2016;6:4166-4178
27. Yu X., Che Z.P., Xu H. Recent Advances in the Chemistry and Biology of Podophyllotoxins. Chem Eur J. 2016;10:1002-1006.
28. Kumari A., Singh D., Kumar S. Biotechnological interventions for harnessing podophyllotoxin from plant and fungal species: Current status, challenges, and opportunities for its commercialization. Crit Rev Biotechnol. 2017;37:739-753.
29. Baldi A., Jain A., Gupta N., Srivastava A.K., Bisaria V.S. Co-Culture of *Linum album* Cells and *Piriformospora Indica* for Improved Production of Phytopharmaceuticals. Varma A., Kharkwal A.C (eds.) In: Symbiotic Fungi, Springer-Verlag Berlin Heidelberg, Soil Biology, 2009,18. DOI: 10.1007/978-3-540-95894-9_22
30. Empt U, Alfermann AW, Pras N, Petersen M. The use of plant cell cultures for the production of podophyllotoxin and related lignans. J Appl Bot. 2000;74:145-150.
31. Sheidai M., Ziaee S., Farahani F., Talebi S.M., Noormohammadi Z., Hasheminejad-Ahangarani Farahani Y. Infra-specific genetic and morphological diversity in Linum album (Linaceae). Biologia. 2014;69:32-39. https://doi.org/10.2478/s11756-013-0281-4.
32. Esmaeilzadeh Bahabadi S., Sharifi M., Safaie N., Murata J., Yamagaki T., Satake H. Increased lignan biosynthesis in the suspension cultures of *Linum album* by fungal extracts. Plant Biotechnol Rep. 2011;5:367-373.
33. Sedaghat S., Ezatzadeh E., Alfermann A.W. Podophyllotoxin from suspension culture of *Linum album*. Nat Prod Res. 2008;22: 984-989. doi: 10.1080/14786410701654685. PMID: 18629714.