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Tissue Culture, *In Vitro* Organogenesis and Regeneration of *Plantago lanceolata*

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Abstract
Introduction: *Plantago lanceolata* is one the most important species of *Plantago* genus and has valuable medicinal secondary metabolites.
Materials and Methods: The effect of different factors on germination of *P. lanceolata* seeds was studied and leaf and root explants of *in vitro* growth seedling were cultured on Murashige and Skoog (MS) medium supplemented with combination of 6-benzylaminopurine (BAP) or thidiazuron (TDZ) (0, 0.5, 1, 1.5, 2 and 3 mg/L) and auxins: α-naphthaleneacetic acid (NAA) (0, 0.2, 0.5, 1 mg/L), indole-3-acetic acid (IAA) (0.1, 0.5, 1, 1.5 mg/L) or indole-3-butyric acid (0.5, 1, 1.5, 2 mg/L) (IBA) at different concentrations.
Results: The results showed that cold pre-treatment, daylight and 1/8 MS salt concentration are more suitable for high germination. The best shoot organogenesis rate (95%) in leaf explants was observed in 1:1 mg/L, and 1.5:1 mg/L TDZ: IBA. The highest percentage of shoot organogenesis (100%) was observed in most of the plant growth regulator (PGR) treatments in root explants. About 58.67 and 60 shoot numbers obtained with 2 mg/L TDZ in leaf and 1:1.5 mg/L BAP: IBA in root explants, respectively.
Conclusions: It can be suggested that the best shoot organogenesis and proliferation medium is MS basal medium containing cytokinin TDZ and auxin IBA in comparison with other hormone compounds on leaf and root explants. The result behind this fact is high callus induction and regeneration potential of explants in all concentrations.
Keywords: Callogenesis, Direct Regeneration, Thidiazuron, Tissue Culture
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Introduction

Plantago is one of the oldest medicinal plants that belongs to the *Plantaginaceae* family. It has been used widely in traditional medicine due to its various chemical compounds including alkaloids, caffeic acid derivatives, coumarins, fats and oils, flavonoids, iridoids, mucilage, polysaccharides, sterols and volatile substances.[1] *P. lanceolata* has also been reported as an expectorant,[2] with anesthetic, anti-viral, anti-inflammatory, astringent, anthelmintic, analgesic, analeptic, anti-histaminic, anti-rheumatic, anti-tumor, anti-ulcer, diuretic, and hypotensive properties.[3,4]

Budzianowska propagated *P. lanceolata* by direct organogenesis of leaf and roots explants on MS medium supplemented with kinetin (Kin) (2 mg/L) and 3-indole acetic acid (IAA) (2 mg/L) and micropropagated plantlets were rooted with Murashige and Skoog (MS) medium plus 1 mg/L IAA. Callus induction and growth of leaves and roots were obtained on MS without NH_4NO_3 plus 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L Kin.[2] In another study, shoot regeneration produced from hypocotyl and cotyledon explants in MS medium supplemented with 6-benzylaminopurine (BAP) (0.75 mg/L) plus α-naphthaleneacetic acid (NAA) (0.2 mg/L) and micropropagation done on MS medium supplemented with thidiazuron (TDZ) (0.1 mg/L) and 3-indole butyric acid (IBA) (0.18 mg/L) and root induction was performed in MS medium supplemented 0.2 mg/L Kin, 0.2 mg/L IBA and 0.5 mg/L NAA.[5]

There is a lot of progress in molecular regulation of biosynthesis pathways of secondary metabolites. The efforts in these research are restricted by the lack of efficient protocols for genetic transformation. Developing genetically transformed plants with high content of interested compounds can be achieved by introducing genes encoding enzymes regulating the biosynthetic pathway. For successful plant transformation, an efficient protocol for *in vitro* tissue culture and plant regeneration is necessary. So, the purpose of this study is to introduce a simple and valuable protocol

for callus induction, *in vitro* shoot organogenesis and plant regeneration of this valuable medicinal plant.

Materials and Methods

Plant Materials

The seeds of *P. lanceolata* were provided from Gene Bank Research Institute of Forests and Rangelands of Iran. Seeds were washed three times with sterile distilled water and were then surface sterilized with 70% ethanol 30-60 seconds and then different percentages of sodium hypochlorite (from 2.5% to 10%) plus one drop of Tween-20 were added for 5-20 minutes and were again washed with distilled water for three times.

Germination Experiments

For pre-treatment, sterile seeds were placed on filter paper and moistened with 10 ml of 0.1 mM Gibberellic Acid (GA_3), 1 mM potassium nitrate (KNO_3) and sterile distilled water as a control for 24 hours in dark and was then cultured in MS placed in a growth chamber at 25°C. The pH of MS medium was adjusted between 5.7 and 5.8 before autoclaving at 121°C and 1.5 kg cm^{-1} pressure for 20 minutes.[6]

For cold pre-treatment, seeds were placed at 4°C for 24 hours in the dark and were then cultured in MS medium.[7] To study the effect of daylight on germination, cold pre-treatment seeds were cultured on agar solidified MS medium and were divided into daylight 16 hours photoperiod at a light intensity of 45 µ $mol.m^{-2}.s^{-1}$ emitted by cool-white fluorescent tubes under 22 ± 2°C temperature, 70 ± 5% relative humidity condition and full darkness conditions.[8] Cold pre-treatment seeds were cultured in MS medium with different salt concentrations (1, ½, ¼, ⅛) and sterile distilled water plus agar for 4 weeks with exposed to daylight (16 hours). Germination rates were recorded from the first week to the fourth week.

Calculation Method of Timson Index

The modified Timson index was applied to calculate the germination rate; velocity: G/t; G is the sum of the percentage of seed germination at 2-day intervals in a period of 28-days, and *t* is total germination period.[9]

In Vitro Culture

Based on germination test results, cold pre-treatment seeds were cultured in ⅛ MS medium with 8 g/L agar in jars and were incubated for 4 to 8 weeks at 16 hours photoperiod. Leaf and root explants from seedlings were cultured in MS medium supplemented with combination of cytokinins: BAP or TDZ and auxins: NAA, IAA or IBA at different concentrations. Concentrations of both cytokines were 0, 0.5, 1, 1.5, 2 and 3 mg/L and for auxins: 0, 0.2, 0.5, 1 mg/L (NAA); 0.1, 0.5, 1, 1.5 mg/L (IAA) ; and 0.5, 1, 1.5, 2 mg/L (IBA). The explants were transferred to fresh medium every 2 weeks to avoid browning and towards shoot organogenesis.

Statistical Analysis

Factorial experiment based on a completely randomized design was used with three replicates. The percentage of callogenesis, regeneration and shoot number were recorded. The data were subjected to analysis of variance test and the means were compared using Tukey test by SPSS 21 version and $P<0.01$ was considered as statistically significant.

Results

Germination of Plantago lanceolata Seeds

For *in vitro* culture, seeds must be disinfected. In this study, the effect of 70% ethanol and sodium hypochlorite was tested at different concentrations on the seeds and the best result was obtained with 70% ethanol 30sec and 5% sodium hypochlorite for 10 minutes plus one drop of Tween-20. Some researchers use many different materials, including calcium hypochlorite with 70% ethanol from 30 sec to 2 min,[2] mercuric chloride (0.05%-0.1%) for 2-10 min[10-12] and sodium hypochlorite (from 3.6 to 5.25%) for 10-30 minutes.[13]

Germination of *P. lanceolata* seeds were examined with different pre-treatments (GA_3, KNO_3, Cold). Results showed that the effect of cold pre-treatment is more effective than GA_3 and KNO_3.

In the first week, the higher germination percentage (78%) was observed in cold pre-treatment. The GA_3 pre-treatment effect (46.67%) was more effective than KNO_3 (35.83%) whereas it was less than control (distilled water) (53.84%) (Figure 1A).

The effect of daylight on seeds germination were also investigated. Results revealed that daylight condition has a significant effect on both seeds germination and the speed of germination, which indicate the positive effects of light on germination and plant growth (Figure 1B).

In the next step of germination experiment, cold pre-treatment seeds were cultured on MS medium containing different salt concentrations (1, ½, ¼ , ⅛). Media with less salt concentration showed a faster germination in the first week of incubation. About 85.71% of germination was attributed to the ⅛ MS medium. After three weeks, all four media, with the exception of the whole MS had 100% germination. The germination rate in sterile distilled water and agar, in the first week, was 80% (Figure 1C).

Finally, the seed germination rate index of *P. lanceolata* was measured in different pre-treatments (Table 1).

Callogenesis and Shoot Organogenesis

After 2-3 subcultures, the callus appeared on most of the explants. The highest callus induction rate (100%) was observed in leaf explants in 1:0.5, 1:1, 1.5:0.2, 2:0.2 TDZ:NAA (Figure 2A), 1:0.5; 1:1; 1:1.5, 1.5:0.5; 1.5:1; 1.5:1.5, 2:0; 2:0.5 mg/L TDZ: IAA (Figure 2B) and 1:1.5; 1:2, 1.5:1.5, 2:2 mg/L TDZ:IBA (Figure 2C) as well as, callus induction rate in BAP (NAA, IAA and IBA) is shown (Figure 3A, B and C) . In root explants,100% callus induction was observed in lots of the plant growth regulators (PGRs) treatments.

The explants with direct regeneration were transferred to

Table 1. Index of Germination Rate of *Plantago lanceolata* Seeds at GA_3, KNO_3, and Cold Treatments

Incubation	Control	GA_3(mM)	KNO_3(mM)	COLD
1 days	46±6.2	42.85±4.6	32.22±3.8	46.70±5.7

Mean of three replications ± standard deviation.

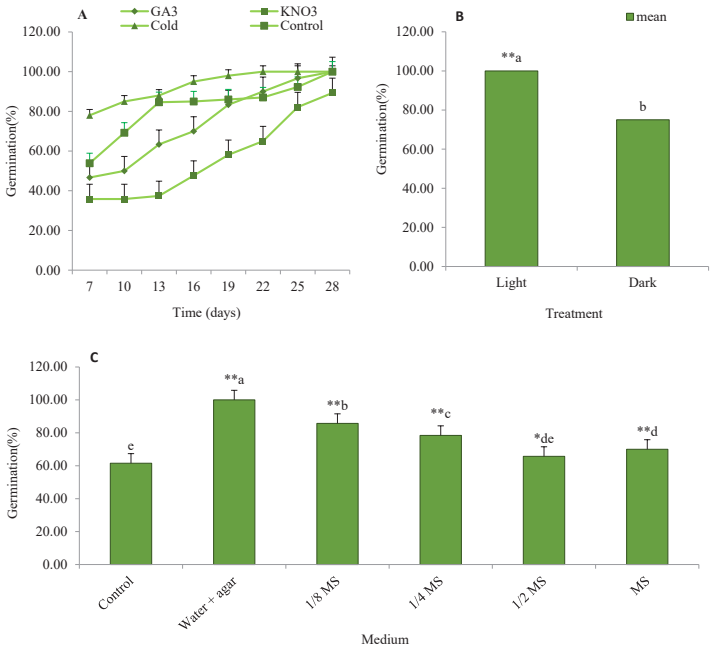

Figure 1. Effect of Different Pre-treatments (A), Daylight (B) and MS Salt Concentration (C) on Seed Germination of *Plantago lanceolata*. Values are as mean ± SD. Mean comparison and mean difference was performed using Tukey (HSD) at the **0.01 and * 0.05 levels.

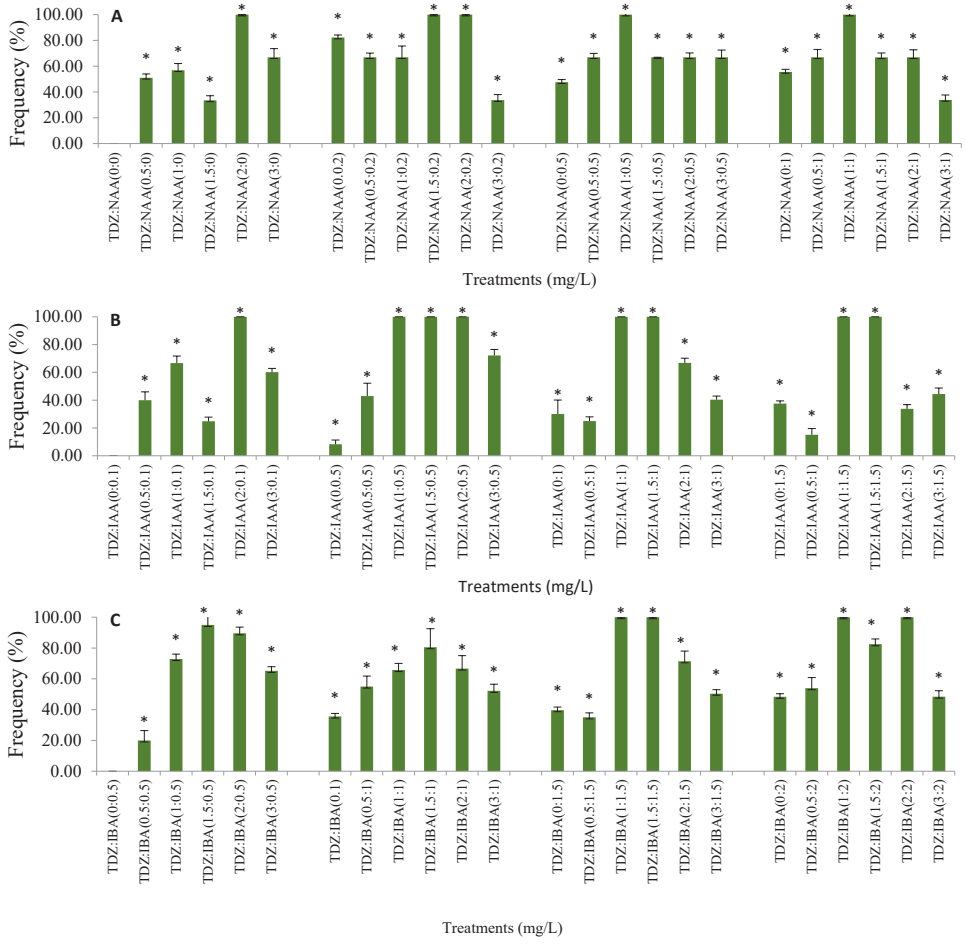

Figure 2. Frequency of Callus Induction of Leaf Explant of *Plantago lanceolata* in TDZ Cytokinin Hormone With Different Auxin Treatment. * indicated the significantly different at the 0.05 level.

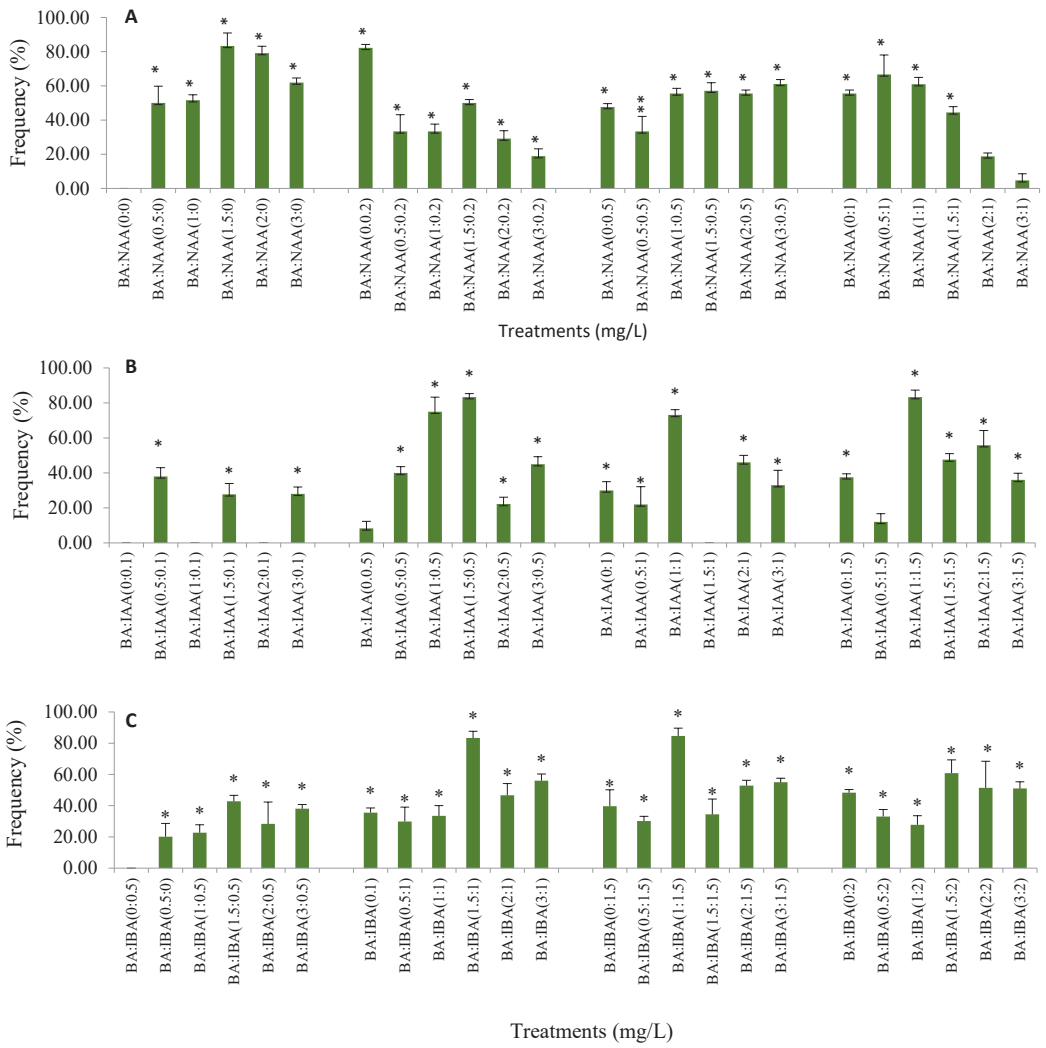

Figure 3. Frequency of Callus Induction of Leaf Explant of *Plantago lanceolata* in BA Cytokinin Hormone With Different Auxin Treatment. * indicated the significantly different at the 0.05 level.

MS medium without PRGs and callus were transferred to MS medium supplemented with 0.5 mg/L (TDZ or BAP) for adventitious shoot induction (Figure 4).

Shoot organogenesis rate (94, 95 and 92%) was detected in leaf explants in MS medium containing 1:1; 1.5:1 mg/L TDZ: IBA (Figure 5C) and 1.5 mg/L BAP (Figure 6A), respectively. A low rate of shoot organogenesis (12%) was observed in combinations of 0.5:1.5 mg/L BAP and IAA (Figure 6B). Most of the root explants were regenerated. The maximum percentage of shoot organogenesis (100%) in root explants was detected in most of the tested PGRs treatments and the minimum regeneration (36%) was observed in media containing 0.5 mg/L IAA.

The high frequency of shoot number (58.67 and 60) was obtained in the media containing 2 mg/L TDZ in leaf explants and 1:1.5 mg/L TDZ: IBA in root explants, respectively (Table 2 A and B).

The results of the present showed that the best shoot organogenesis was achieved in different combinations of TDZ: IBA in explants. One of the main findings of this study is the treatment of explants by BAP which showed that the regeneration is more direct. Direct regeneration of plant is very important since it can be used for several purposes such as plant propagation and genetic transformation. Treatment by TDZ leads to more indirect regeneration.

For root induction, regenerated shoots were transferred into MS agar-solidified medium without growth regulators or supplementation with different auxins after 2 weeks. The root induction in medium containing NAA, IAA and IBA (0.1 and 0.5 mg/L) was performed and results indicated that the best auxin for root induction was 0.5 mg/L IBA, since the other hormones showed a small amount of callus formation. The rooted seedlings were transferred to MS PGRs-free medium and were then successfully transplanted to sterile 80% peat moss and 20% perlite mix in the pot. Seedlings were watered with Hoagland solution and distilled water. The survival rate of plantlets was 100%. The plantlets were acclimatized in the greenhouse (Figure 7).

Table 2. The Highest Regeneration Frequency in Leaf Explants in Different Hormonal Compositions Along With the Percentage of Callus Formation and the Number of Branching Associated With Each Regeneration

Cytokinins A	Auxin	Concentration (Cytokinin: Auxin)	Callus (%)	Regeneration (%)	IR (%)	DR (%)	Adventitious Shoot
BA	NAA	1.5:0	83.33±3.2	92.59±3.3	75.92	16.66	18.45±4.3
	IAA	1.5:1.5	47.61±2.9	70.83±4.5	52.38	18.45	15±2.8
	IBA	1:1.5 and 1.5:1	84.62±3.8; 83.33±4.2	64.62±2.1; 63.33±2.8	58.2; 60.12	4.42; 3.21	15.82±3.3; 16±4.6
TDZ	NAA	2.0.2	100±0	77.77±4.62	77.77	0	13.5±1.5
	IAA	1.5:0.5	100±0	85.71±1.9	85.71	0	12.66±2.2
	IBA	1:1 and1.5:1	65.71±5.7; 80.55±2.7	95±4.2; 94.44±5.5	68.57; 88.88	27.14; 5.55	31.08±1.7 12.05±2.5
Cytokinins B	**Auxin**	**Concentration (Cytokinin: Auxin)**	**Callus (%)**	**Regeneration (%)**	**IR (%)**	**DR (%)**	**Adventitious Shoot**
BA	NAA	1.5:0	16.66±1.8	100±0	16.66	83.33	56.3±2.5
	IAA	2:0	0±1	100±0	0	100	36±3.8
	IBA	1.5:0.5	50±2.1	84±3.5	32	52	42±4.2
TDZ	NAA	1: 1	100±0	100±0	100	0	50±2.6
	IAA	1: 1.5	100±0	100±0	100	0	38.5±2.6
	IBA	1.5:2	100±0	100±0	100	0	60±4

A: The highest percentage of regeneration and callogenesis and adventitious shoot number associated with each regeneration in leaf explant of Plantago lanceolata and B: The highest percentage of regeneration and callogenesis and adventitious shoot number associated with each regeneration in root explant of Plantago lanceolata IR: Indirect Regeneration, DR: Direct Regeneration, the value is mean of replication ± Standard Deviation (Mean ± SD)

Discussion

In most plants, seeds are germinated without any pre-treatment but in some cases, GA_3 is used to break seed dormancy and increase the seed germination rate.[14] In *Plantago major*, KNO_3 (10^{-3} M) has been found to be effective in undesirable light conditions.[15] In the current study, at beginning of the experiment, cold pre-treatment has increased the germination rate in comparison with all pre-treatments. In some investigations, it revealed that cold stratification of *P. lanceolata* seeds can improve the germination frequency.[15] It was also observed that the growth of plants after the cold pre-treatment had increased. Researchers have found that low-temperature pre-treatment can improve germination of seeds and also increase the induction of root and shoot on callus cultures.[16]

Relatively high germination in darkness can be due to the breaking of seed dormancy resulted from cold pre-treatment. Dark conditions are used to germinate *Plantago maritima*[17] and *Plantago ovata*[12] seeds. According to Pons, the germination of *P. lanceolata* in dark was 50% and less than the daylight conditions (100%) but he pointed out to this point that this species has a seasonal change in dormancy with the highest germinability, whether in light or dark, in spring, and the lowest in autumn.[18] Our results are consistent with Pons

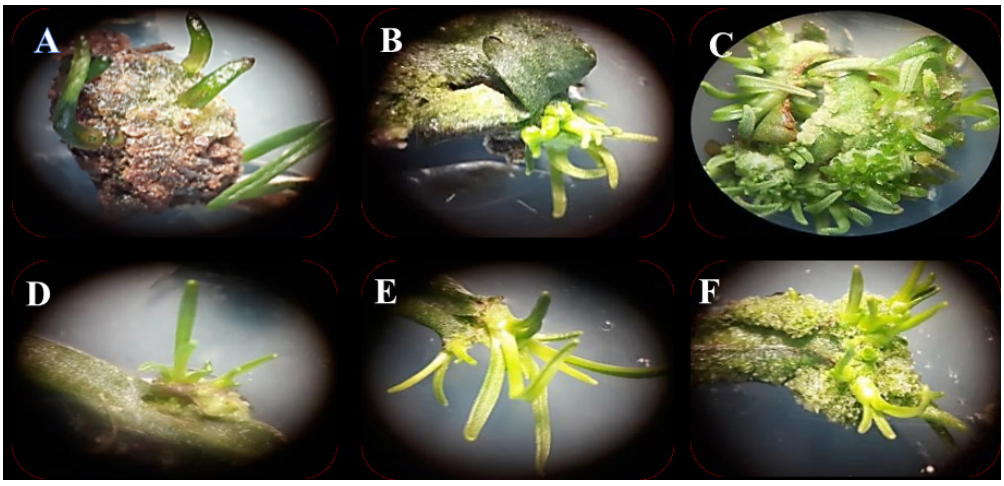

Figure 4. Indirect (A-C) and Direct (D-F) Shoot Organogenesis on Leaf Explants of *Plantago lanceolata*.

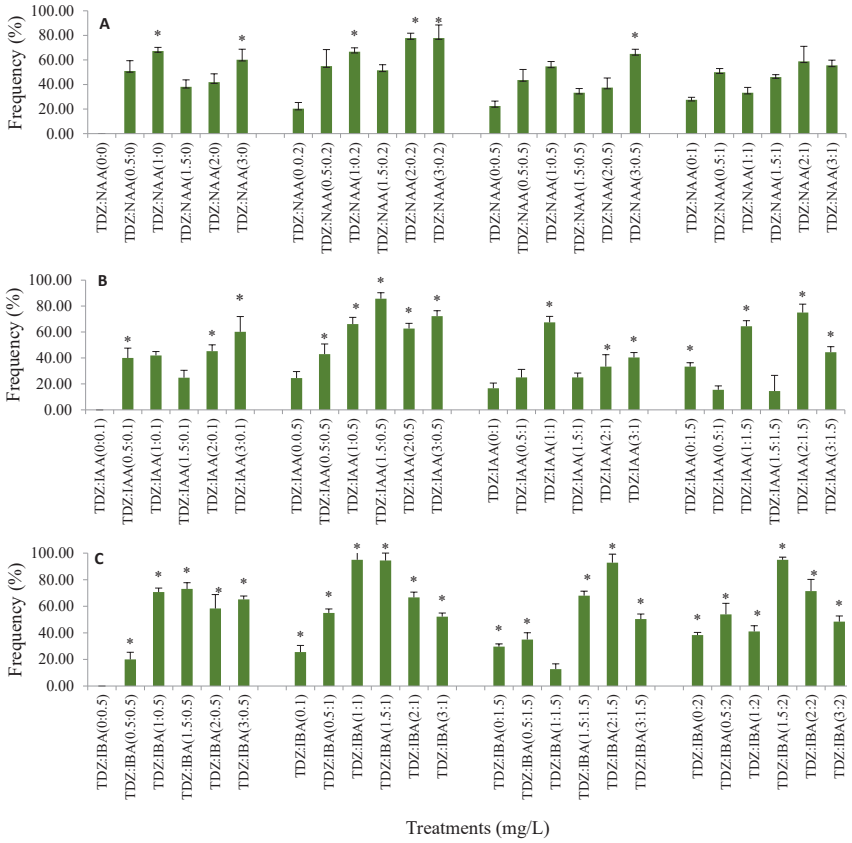

Figure 5. Frequency of Shoot Organogenesis of Leaf Explant of *Plantago lanceolata* in TDZ Cytokinin Hormone With Different Auxin Treatment. * indicated the significantly different at the 0.05 level.

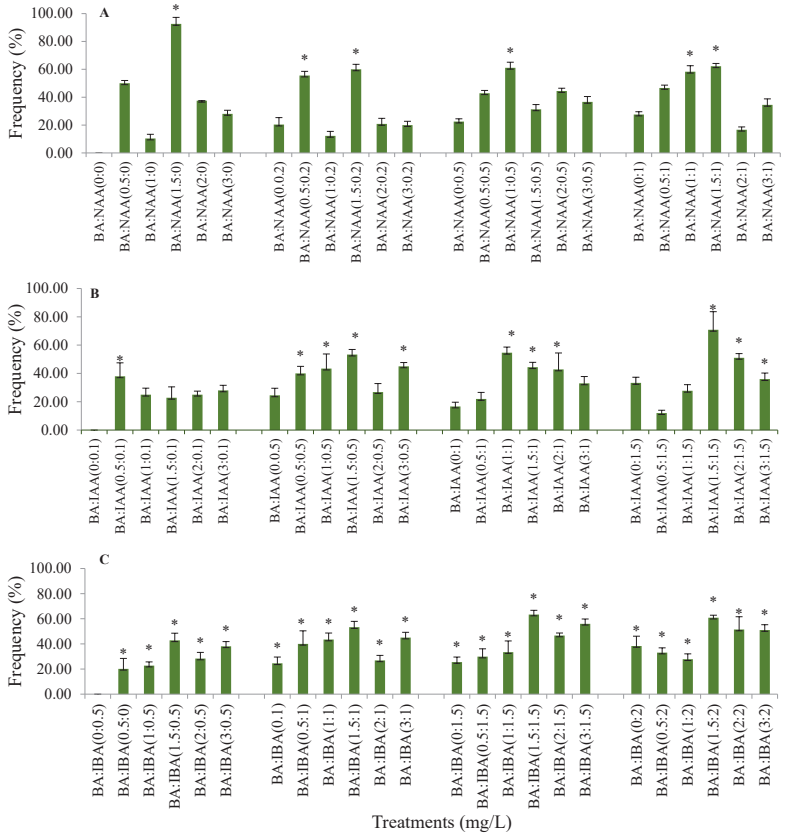

Figure 6. Frequency of Shoot Organogenesis of Leaf Explant of *Plantago lanceolata* in BA Cytokinin Hormone With Different Auxin Treatment. * indicated the significantly different at the 0.05 level.

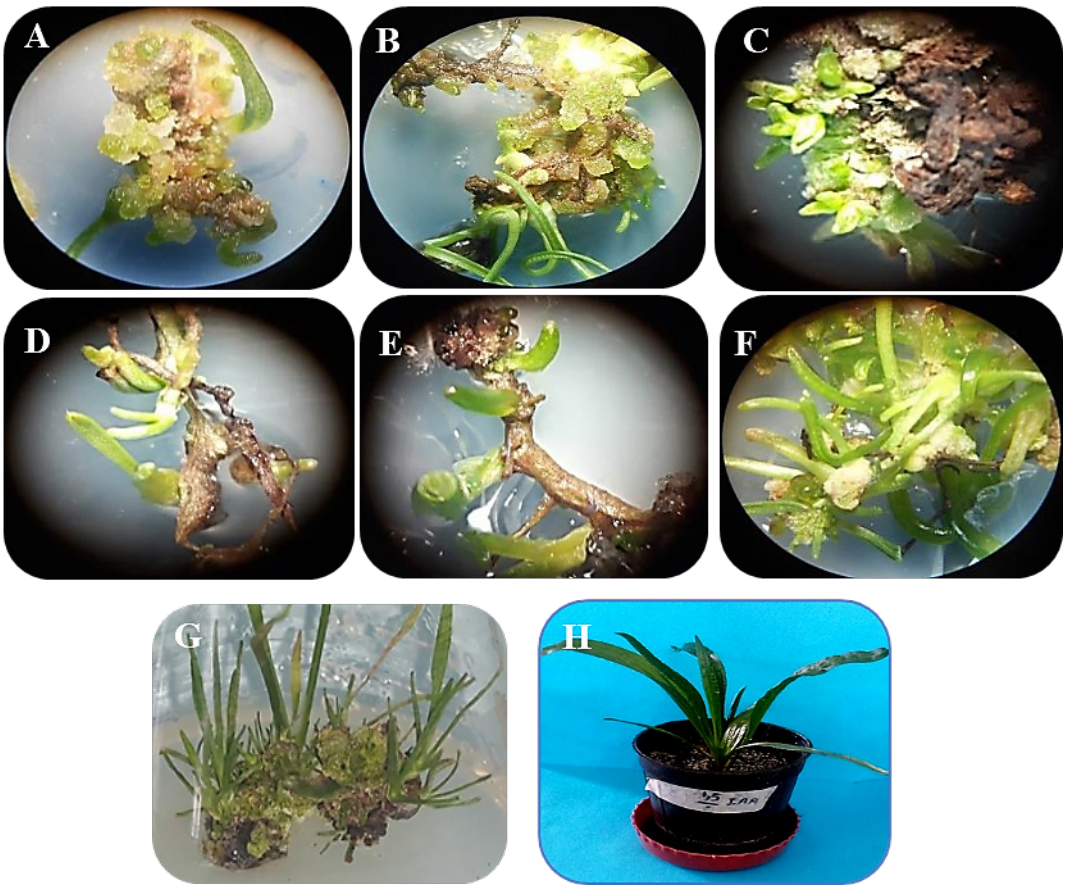

Figure 7. Indirect (A-C) and Direct (D-F) Shoot Organogenesis in Root Explants of *P. lanceolata,* Rooted Plantlets (G) and Acclimatized Plant (H).

research and we also found that daylight conditions are more beneficial not only for germination of *P. lanceolata* but also for plant growth in non-seasonal conditions.

The high rate of root induction in shoot organogenesis can be due to the high contents of endogenous growth regulators.[19] It has been shown that the directly regenerated seedling acclimatized without treatments with rooting hormone.[20] Therefore, we also observed that the most regenerated plantlets can be rooted in hormone-free medium except for TDZ treatments. As previously stated, TDZ completely inhibited root development.[5] Experimental glasshouses should have a high relative humidity in order to maximize seed germination and rooting.[21,22] In this study during the adaptation period, the plant needed regulated temperature and adequate humidity at least for two weeks. *P. lanceolata* contain important secondary metabolites such as iridoids (aucubin and catalpol), thus *in vitro* cultures could be important for the production of these compounds.

Conclusions

The present protocol has introduced a simple and rapid tissue culture system for high-frequency *in vitro* shoot organogenesis and regeneration of *P. lanceolata*. The results showed that MS basal medium containing cytokinin TDZ and auxin IBA is the best hormonal compounds for the regeneration and propagation of leaf and root explants in comparison with other hormonal compounds. Moreover, it was found that *P. lanceolata* has a high regeneration potential and its leaf and root explants can be regenerated directly and indirectly. Tissue culture of this medicinal weed which is widespread in Iran can be of great help to further application of *Plantago* sp. It can also be an effective method to produce more secondary metabolites. Hence, this research group is focusing on the investigation of tissue culture plantlets of *Plantago* spp. to produce the effective medicinal compounds (e.g. aucubin and catalpol) and genetic transformation.

Authors' Contributions

SRH carried out the experiments as a PhD student. AS, KB, and HD supervised the study. All authors contributed to the study. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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