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PLANT TISSUE CULTURE

Efficient *in vitro* organogenesis, micropropagation, and plumbagin production in *Plumbago europaea* L.

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Abstract

In this study, an efficient method for *in vitro* regeneration of *Plumbago europaea* was developed using direct and indirect organogenesis. Accordingly, micropropagation and regeneration were obtained on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of plant growth regulators. The effects of explant type and plant growth regulators on shoot organogenesis of *P. europaea* were evaluated. For the nodal explants, MS medium containing 0.5 mg/l TDZ (11.62 shoots per node) was the best medium for high frequency of micropropagation. In comparison, the highest percentage of direct organogenesis (70%) and number of shoots per explants (14.6) were acquired for the internode explants using 0.5 mg/l TDZ and 0.1 mg/l IAA. The obtained data revealed that TDZ is the most effective cytokinin for the direct shoot organogenesis. The highest indirect organogenesis rate was observed using 2 mg/l BA and 0.1 mg/l NAA for the internode explant. The maximum number of roots was distinguished on ½ MS medium containing 0.5 mg/l IBA (6.42). The rooted plantlets were gradually hardened and acclimatized under *ex vitro* conditions. As an important outcome, the active compound plumbagin was found mainly in the root tissues of the micro-propagated and regenerated plantlets. Taken all together, this study achieved a successful protocol for *in vitro* regeneration of *P. europrea* and could be considered for large-scale multiplication of this important medicinal plant.

Keywords Organogenesis · *Plumbago europaea* · Regeneration · Thidiazuron

Introduction

Plumbago europaea L. (Plumbaginaceae) is a valuable medicinal plant which is native to the Mediterranean region, Europe, north Africa, and south west Asia. It is the only species of the genus *Plumbago* growing wild in Iran (Rechinger and Schiman-Czeika 1974). Roots of *P. europaea* are known to have the capacity to produce large amounts of plumbagin as a bioactive naphthoquinone (Al-Nuri *et al.* 1994; Muhammad *et al.* 2009; Iwashina 2013). Plumbagin has been reported to possess antimicrobial, anticancer, antifertility, pesticidal, and antimalarial activities (Kubo *et al.* 1983; Likhitwitayawuid *et al.* 1998; Ding *et al.* 2005; Kuo *et al.* 2006; Nair *et al.* 2016).

Propagation of *P. europaea* by seeds is difficult due to poor seed quality, lower germination rate, and low seedling survival under natural field conditions (Chaplot *et al.* 2005). Plant cell, tissue, and organ cultures can be regarded as suitable alternative approaches to conserve many valuable plants and propagate sufficient amount of plants. *In vitro* propagation approaches, as asexual propagation methods, have some superiority over traditional techniques of propagation, including short production time, no dependence on season constraints, and production of disease-free plantlets. Furthermore, *in vitro* plant propagation requires low amounts of plant material with minor effect on wild populations (Nowakowska *et al.* 2020). Plants propagated *via* tissue culture are genetically identical to the original plant and are uniform in

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size, shape, and yield. Production of specific compounds/proteins using cell and tissue culture systems can be triggered by means of elicitors. Moreover, endangered or threatened species of plants can be cultivated for preservation and other purposes (Bhatia *et al.* 2015).

Plumbago species begin to produce restricted amounts of plumbagin between 2 and 6 yr of age (Kitanov and Pashankov 1994). Therefore, it is necessary to develop an efficient and improved protocol for production of sufficient yield of this bioactive secondary metabolite. Several studies have been conducted for callus induction, organogenesis, and regeneration of *Plumbago* species (Harikrishnan and Hariharan 1996; Selvakumar *et al.* 2001; Das and Rout 2002; Jose *et al.* 2007; Bhadra *et al.* 2009; Gopalakrishnan *et al.* 2009; Haque *et al.* 2012; Chatterjee and Ghosh 2015). *In vitro* propagation of *P. rosea* was previously attained through organogenesis from different types of explants such as leaves, shoot tips, axillary buds, and somatic embryos. However, no report is available on the *in vitro* propagation of *P. europaea.* Therefore, we aimed to identify the most responding explants as well as suitable plant growth regulators and their optimum concentrations for effective *in vitro* regeneration of *P. europaea*.

Cytokinins like BA, kinetin, zeatin, and TDZ may help the cultured tissues to produce adventitious shoots. BA and TDZ have been also used as growth regulator to induce shoots from the explants and for rapid *in vitro* regeneration (Moshtaghi 2020). Although shoot regeneration responses vary depending on the type of explant and cultivar, in most cases exposure of explants to cytokinins may promote shoot production (Panizza and Tognoni 1992; Ghorbani *et al.* 2021). TDZ, a synthetic phenylurea, possesses powerful cytokinin-like activity. It has been frequently used in the *in vitro* plant regeneration systems (Murthy *et al.* 1998; Pourebad *et al.* 2015). The purpose of this study was to determine an efficient and rapid shoot organogenesis protocol for *P. europaea* by investigating the effect of explant types and plant growth regulators.

Materials and methods

Plant material and culture condition The seeds of *P. europaea* were obtained from Research Institute of Forests and Rangelands, Tabriz, Iran. The seeds were surface sterilized and cultured on the MS medium for germination. All the media used in this study were supplemented with 3% (w/v) sucrose and solidified with 0.7% (w/v) agar. The pH of media was adjusted to 5.8. All the cultures were incubated at 25°C and under a 16/8-h light/dark photoperiod in the growth chamber with the light intensity of 40 mmol $m^{-2}s^{-1}$ using cool white fluorescent lamps. Three-wk-old *in vitro* grown seedlings were used as a source of leaf, internode, and nodal explants for shoot organogenesis studies.

In this study, two experiments were designed for propagation purposes. In the first experiment, the effects of BA and TDZ on direct shoot organogenesis were evaluated using nodal explants of *P. europaea*. Nodal (contain auxiliary bud) explants were vertically cultured onto the full-strength MS medium containing the different concentrations of BA or TDZ (0.5, 1, and 2 mg/l). After 3 wk of culture, clusters of multiplied shoots in BA or TDZ containing media were transferred to basal solid MS medium without hormones to eliminate the effects of cytokinins on growth and development of the buds. After 2 wk of shoot elongation, shoots were transferred to root-inducing culture media.

In the second experiment, MS culture medium containing different concentrations and combinations of cytokinins (TDZ and BA) and auxins (NAA and IAA) was used for efficient plant regeneration from leaf and stem (internode) explants. In addition, a hormone-free medium was considered as control. Media were poured in sterilized culture vessels. The leaves and internodes from 3-wk-old plants were cut and cultured on solidified MS medium supplemented with different combinations of IAA, NAA, BA, and TDZ consisting of BA (0.5, 1, 2 mg/l), TDZ (0.5, 1, 2 mg/l), NAA (0, 0.05, 0.1 mg/l), and IAA (0.1, 0.5 mg/l). The explants were incubated under the same condition as for the first experiment. After callus or shoot induction, explants were transferred to MS media without hormones or supplemented with 0.5 or 1 mg/l BA for adventitious shoot induction.

Rooting and acclimatization In order to develop root system, regenerated shoots were excised from the parent plants and transferred to the MS and 1/2 MS media containing different concentrations of NAA or IBA. In most of the treatments, root induction was observed after 7 to 10 d. After root induction, the explants were transferred to the hormone-free media. The frequency and the average number of roots in each treatment were recorded after 4 wk of culture.

The *in vitro* rooted and healthy plants were separated from rooting medium and their roots were washed to remove adhering gel. They were then transplanted to plastic pots containing garden soil and sand at a ratio of 3:1 and covered with the bottle. Plants were covered with transparent plastic bags to ensure high humidity. They were kept in a culture room for 2 wk, and then transferred to the shaded greenhouse until achievement of the appropriate growth. Their survival rate was measured for a period of 4 wk in greenhouse conditions. Each experiment was conducted in three repetitions. The obtained data were analyzed statistically using Duncan's multiple range test.

Quantitative determination of plumbagin In order to examine the ability of roots and aerial parts of micro-propagated

and regenerated plants to produce plumbagin, dried samples were ground to fine powder using a mortar and pestle. Each sample (1 g) was extracted three times with 10 ml of chloroform for 3 d. After passage through a filter paper, the solvent evaporated by a rotary evaporator. The residue was dissolved in 5 ml of methanol and 50 µl of methanolic extract of sample was injected into C8 analytical column (250 mm * 4.6 mm, particle size 5 µm; PerfectSil, MZ-Analysentechnik, Mainz, Germany). A Breeze HPLC system from Waters Corporation (Milford, MA) was used at a wavelength of 270 nm. The mobile phase was a mixture of methanol:water (80:20) and run at the isocratic condition with a flow rate of 0.9 ml min^{-1}. Quantitative analysis of plumbagin was carried out based on the peak area of the sample and the known concentrations of standard. The area under the peaks of plumbagin was integrated and converted to concentration using its calibration curve.

Results and discussion

Tissue culture and micropropagation using nodal explants In the first experiment, nodal explants were cultured on the MS medium supplemented with different concentrations of BA or TDZ. One of the most widely used strategies for micropropagation is axillary bud proliferation, upon which the nodal segments harboring axillary buds are cultured to regenerate the shoots (Nowakowska *et al.* 2020). Organized meristems such as shoot tips and axillary buds are less prone to spontaneous genetic changes because meristems are more resistant to genetic changes than disorganized tissues (Krishna *et al.* 2016).

A rapid *in vitro* propagation using nodal explants was observed in the MS medium containing 0.5 mg/l TDZ with the highest percentage of micropropagation rate (87%) and maximum number of shoots (11.62 shoots per node) (Fig. 1). The nodal explants directly produced multiple shoot buds and developed new plantlets. The medium containing TDZ was more efficient for micropropagation than the medium supplemented with BA (Figs. 1 and 2). Based on previous reports, TDZ was proved to be superior for micropropagation of *Cardiospermum halicacabum* and *P. zeylanica* (Jahan and Anis 2009; Ceasar *et al.* 2013). Micropropagation was not observed on the control medium (without growth regulator), which confirmed the significance of cytokinins in the stimulation of the cell division and organogenesis.

Applying the exogenous cytokinins and removing the apical meristem promote apical dominance reduction, lateral bud break, and adventitious shoot induction in plant tissue culture (Banthorpe *et al.* 1986; Panizza and Tognoni 1992; Babaei *et al.* 2014).

Choosing the optimal concentration and type of plant growth regulator is a critical step for efficient regeneration. In fact, TDZ has been established as a substituted phenyl urea-type cytokinin and an important regulator for morphogenic responses, inducing *in vitro* adventitious bud and shoot organogenesis (Jiang *et al.* 2005; Yucesan *et al.* 2007; Sajid and Aftab 2009). It has been confirmed that the impact of TDZ concentrations can be effectively dependent on the plant species, explant status, and culture condition (Guo *et al.* 2011). Shoot regeneration using TDZ was studied in some species of *Plumbago* (Patidar *et al.* 2013; Sharma and Agrawal 2018). In some cases, the application of TDZ showed negative effects on shoot development and resulted in glassy structure production, stem elongation, and

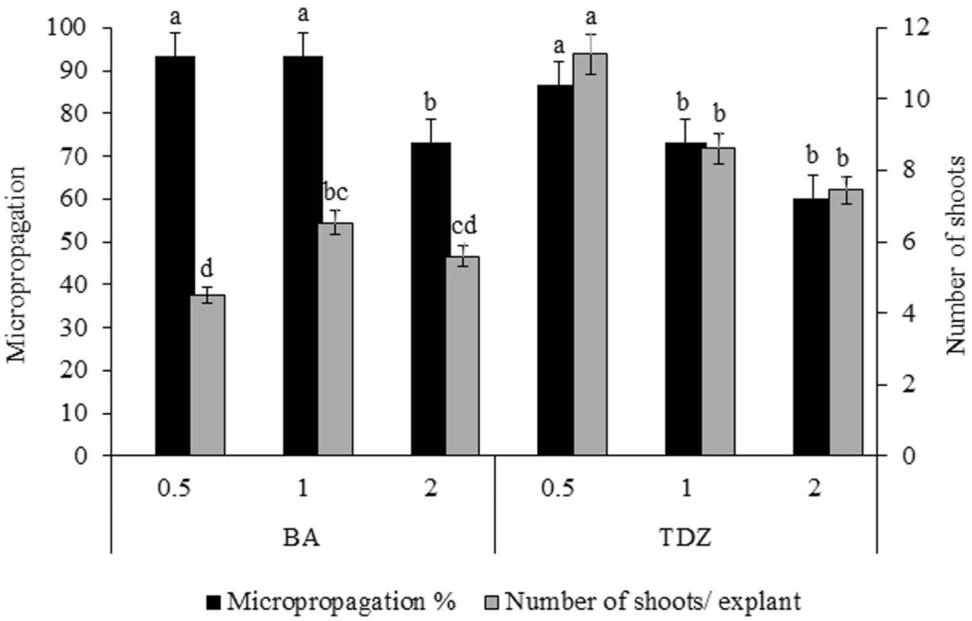

Figure 1. Effects of TDZ and BA on micropropagation and average number of shoots in nodal explants of *P. europaea.* The data represent the mean ± SD of three independent experiments. The means indicated by the same letters are not significantly different by Duncan's multiple range test ($p \leq .05$).

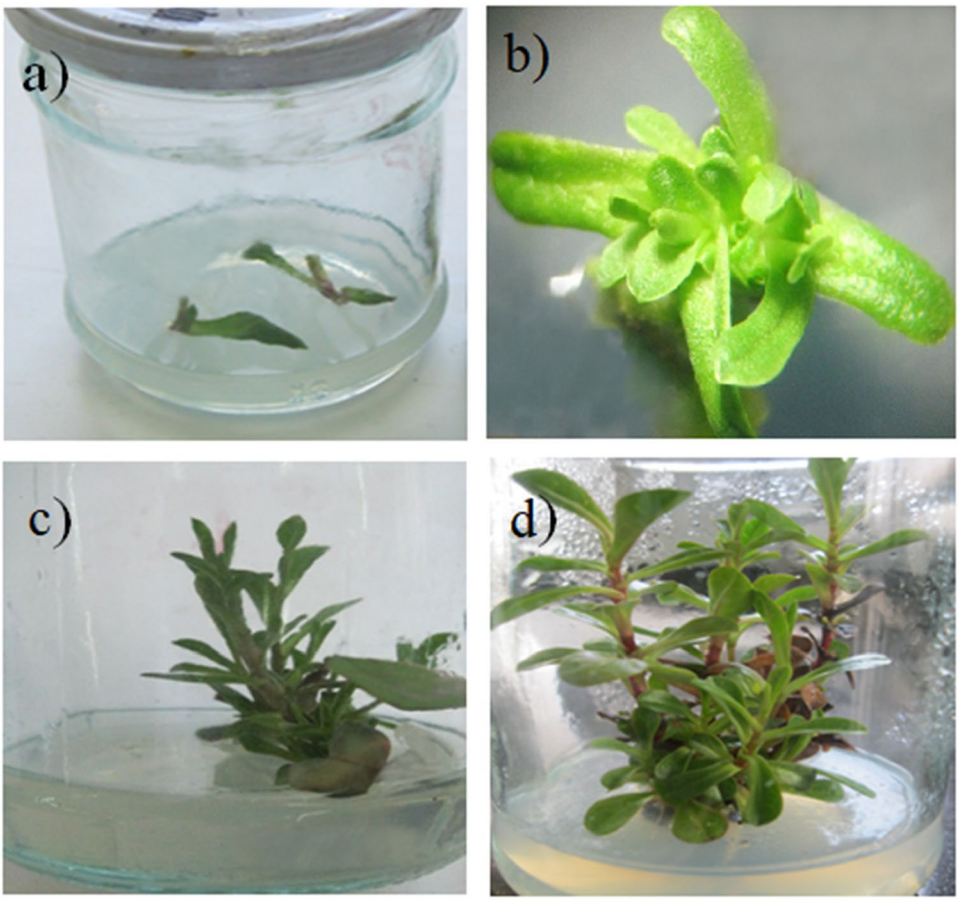

Figure 2. Micropropagation of *P. europaea*. (***a***) Shoot formation from nodal explants on MS medium supplemented with 0.5 mg/l TDZ after cultivation. (***b***) Shoot formation after 2 wk of cultivation. (***c***, *d*)1 wk and 2 wk after transfer to MS medium.

morphology abnormalities (Huetteman and Preece 1993). In the current study, to prevent the adverse effects of long-term exposure to TDZ, explants were transferred onto the TDZ-free MS medium for proliferation, development, and the elongation of the induced shoots. The efficient role of TDZ on micropropagation has been shown for plant species such as rose and *Pterocarpus marsupium* (Barna and Wakhlu 1995; Hussain *et al.* 2007).

Micropropagation through axillary bud proliferation has proven to be a handy tool for the conservation of valuable medicinal plant resources (Pierik 1997; Malathy and Pai 1998;Selvakumar *et al.* 2001; Najaf-Abadi and Hamidoghli 2009; Corral *et al.* 2011; Nowakowska *et al.* 2020). Although the technique of nodal micropropagation has been reported previously for some species of *Plumbago* such as *P. indica* (Gradner 1991) and *P. zeylanica* (Selvakumar *et al.* 2001; Chandravanshi *et al.* 2014), such a procedure has so far not been used for *P. europaea.* In this paper, we present, for the first time, a rapid protocol for the *in vitro* propagation of this plant through axillary bud culture.

Organogenesis and regeneration using leaf and internode explant The results of the second experiment showed that among the 34 hormonal treatments, organogenesis occurred by using 11 and 20 treatments for leaf and internode explants, respectively. No callus induction and regeneration were observed in the control explants. The obtained results revealed that optimal concentration and combination of both cytokinin and auxin had an essential role in the regeneration of shoots from leaf and internode explants of *P. europaea*.

Using NAA + BA and IAA + BA combinations, callus production was established in both types of explants (Fig. 3). The shoots were induced indirectly via callus from internode and leaf segments of plants (Fig. 4). The highest indirect organogenesis rate was observed using 2 mg/l BA and 0.1 mg/l NAA for the internode explant (Figs. 5 and 6).

By application of TDZ + NAA and TDZ + IAA, direct shoot organogenesis occurred (Fig. 7). The highest percentage of direct shoot organogenesis (14.6 seedlings from each explant) was attained by 0.5 mg/l TDZ and 0.1 mg/l IAA in the internode explants (Figs. 5 and 6). TDZ in combination with NAA or IAA displayed the best impact on direct shoot organogenesis from the leaf and internode of *P. europaea*.

Based on the obtained results, significant effects of explant type and plant growth regulator on callus induction and organogenesis of *P. europaea* were confirmed (Figs. 3 and 5). These data were consistent with the former reports showing that culture media containing TDZ in combination

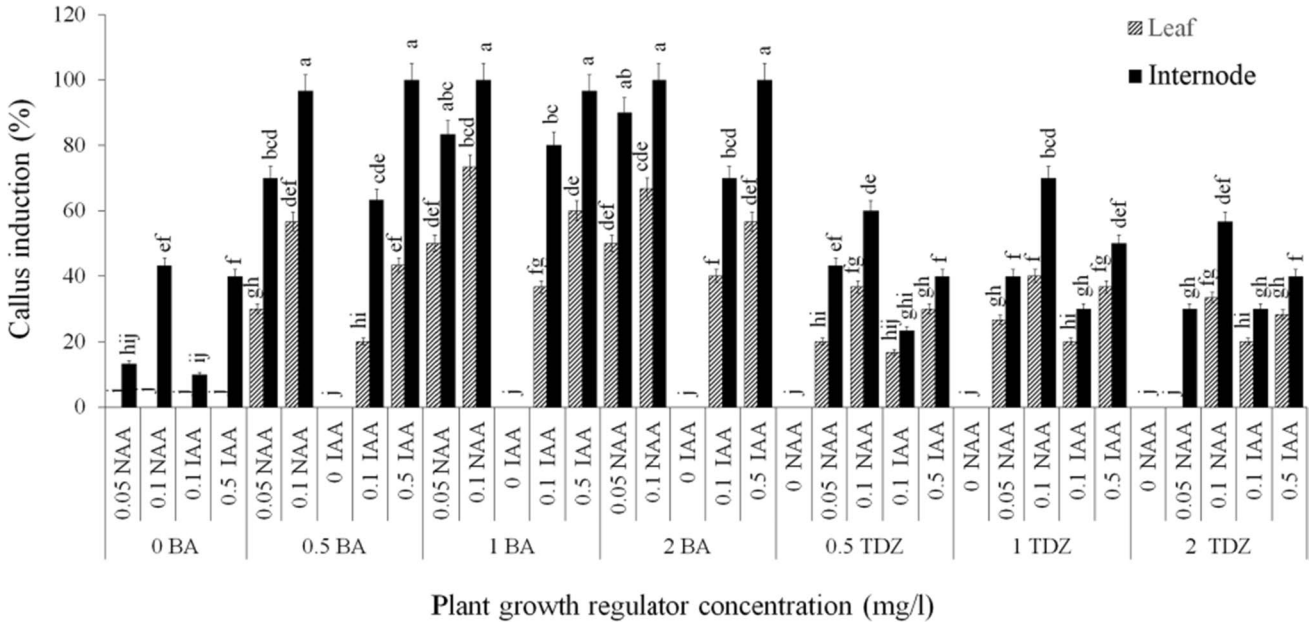

Figure 3. Effects of plant growth regulators on callus induction in leaf and internode explants of *P. europaea*. The data represent the mean ± SD of three independent experiments. The means indicated by the same letters are not significantly different by Duncan's multiple range test ($p \leq .05$)

with IAA exhibited the highest efficiency for the direct organogenesis in different plant species (Kasula *et al.* 2008; Nazari *et al.* 2016). Cytokinins are the most important growth regulators for plant regeneration (Song *et al.* 2011). The type of cytokinin could be regarded as one of the most important factors affecting the organogenesis of shoots. The mechanism of TDZ action has not yet been fully elucidated. Based on a suggestion, TDZ may induce organogenesis by direct stimulation of tissue. In contrast, the second conception expresses that TDZ stimulates intrinsic cytokinins and thereby facilitates the organogenesis of shoot (Huetteman and Preece 1993).

Other important factors including plant genotypes, type of explant, growth regulator combination, and medium composition play important roles in the efficiency of *in vitro* organogenesis (Tomsone and Gertnere 2003). Our results confirmed the preceding reports indicating the significant role of plant growth regulators and type of explants on regeneration of shoots (Asghari *et al.* 2012). Maximum organogenesis rate was observed in stem explants, and therefore stem explants were more efficient than leaf explants for callus formation and, shoot organogenesis (Figs. 5 and 6). The direct shoot organogenesis of *P. zeylanica* was studied and the outcomes confirmed that leaves were the best explants for the organogenesis (Rout *et al.* 1999). These results were in fair agreement with the findings of previous reports (Türker *et al.* 2010; Biswas *et al.* 2012). It was also observed that leaf explants showed the lower callus induction in *Solanum tuberosum* compared to the stem explants (Kumlay and Ercisli 2015). The diverse responses of the explant types may be due to the differences in the levels of the endogenous hormones, in the meristematic activity of the cells, in the physiological condition of explants, and in the expression levels of genes encoding hormone receptors (Close and Gallagher-Ludeman 1989; Kaul *et al.* 1990; Sujatha and Mukta 1996; Kaewpoo and Te-chato 2009). Even in a precise genotype, plant growth regulators may exhibit different effects based on the genotype of cells (Annadana *et al.* 2000).

Rooting and acclimatization Regenerated shoots (4–5 cm) were transferred onto the half- and full-strength MS medium containing NAA and IBA. Consequently, maximum number of root per shoot (6.3±0.4) occurred in 1/2 MS medium containing 0.5 mg/l IBA (Figs. 8 and 9). IBA is a common plant growth regulator suggested for root induction specially in low concentrations in many plants such as bamboo (Alagumanian *et al.* 2004), *Jatropha curcas* (Singh *et al.* 2010), *Cannabis sativa* (Lata *et al.* 2010), and *Stevia rebaudiana* (Alhady 2011). These data were also in line with the former finding, which showed a better rooting response on 1/2 MS than MS medium for *P. zeylanica* (Ceasar *et al.* 2013). The increase in root number and length is very important for the acclimatization to *ex vitro* conditions, as well as for the water and nutrient uptake of plantlets (Sanavy and Moeini 2003). The hardening of plantlets was important for the

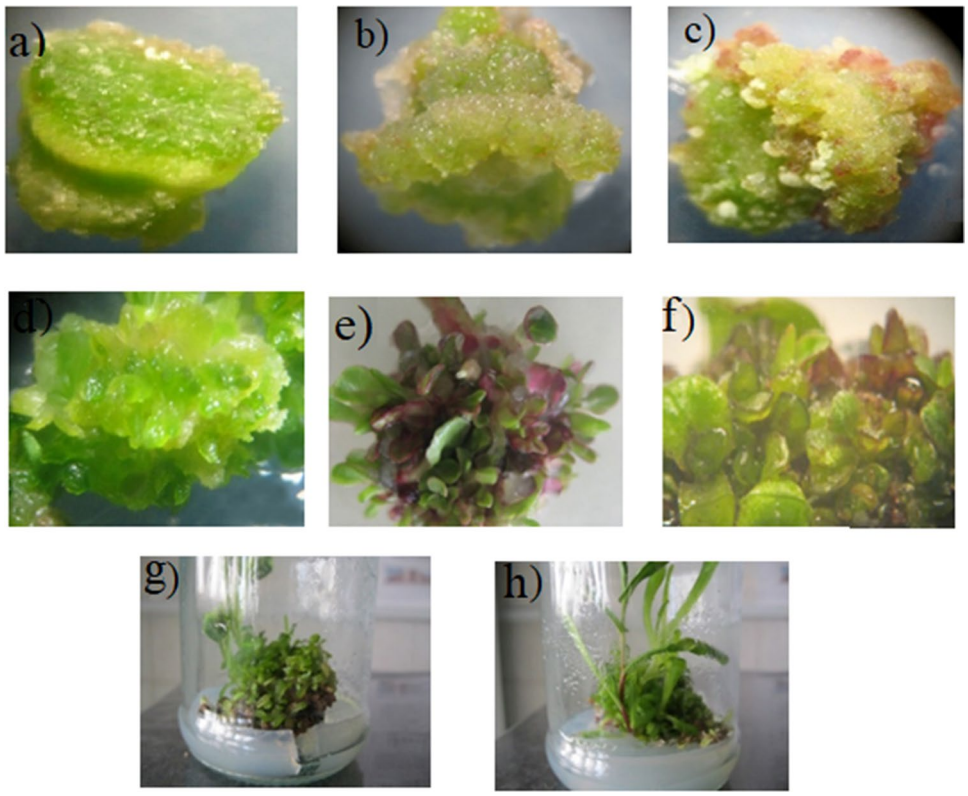

Figure 4. Indirect shoot organogenesis process. (***a–c***) Callus induction from internode explants on MS medium containing 2 mg/l BA and 0.1 mg/l NAA. (***d–h***) Elongation of shoots after transfer to hormone-free MS medium.

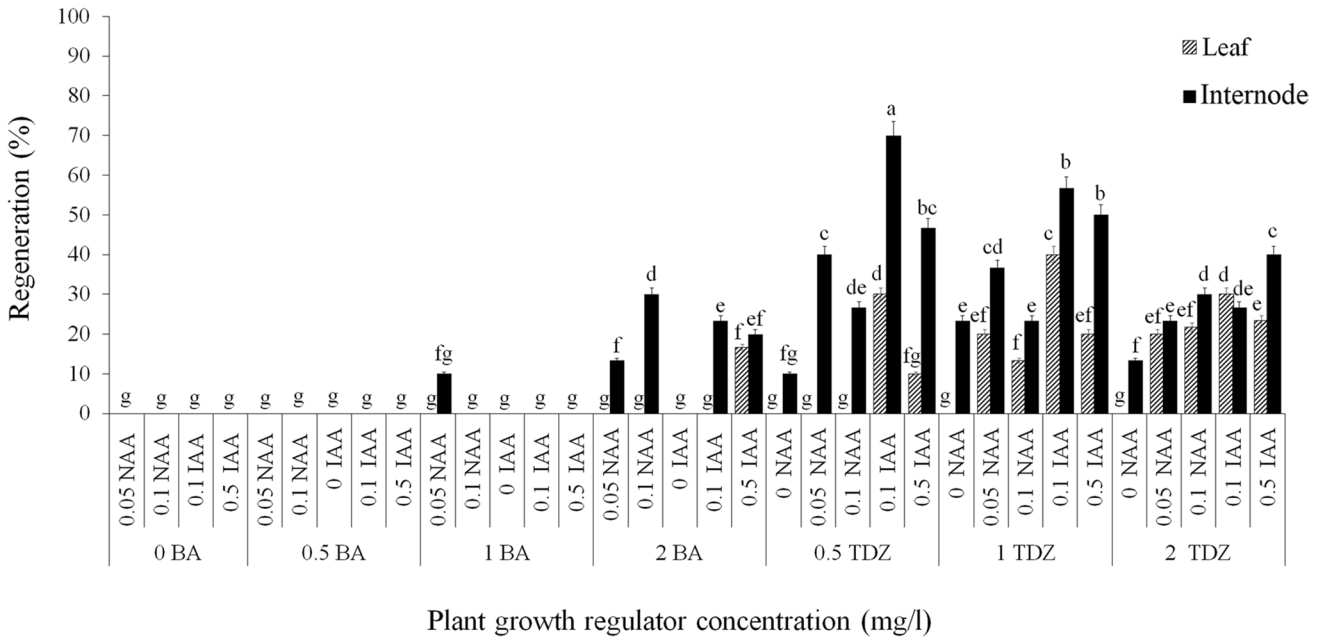

Figure 5. Influences of plant growth regulators on regeneration rate of leaf and internode explants of *P. europaea*. The data represent the mean ± SD of three independent experiments. The means indicated by the same letters are not significantly different by Duncan's multiple range test ($p \leq .05$).

transplantation of plantlets to the field. In our study, plantlets were transferred into pots covered by plastic for 2 wk to maintain 100% relative humidity. After 2 wk, the humidity was gradually decreased by opening and finally removing

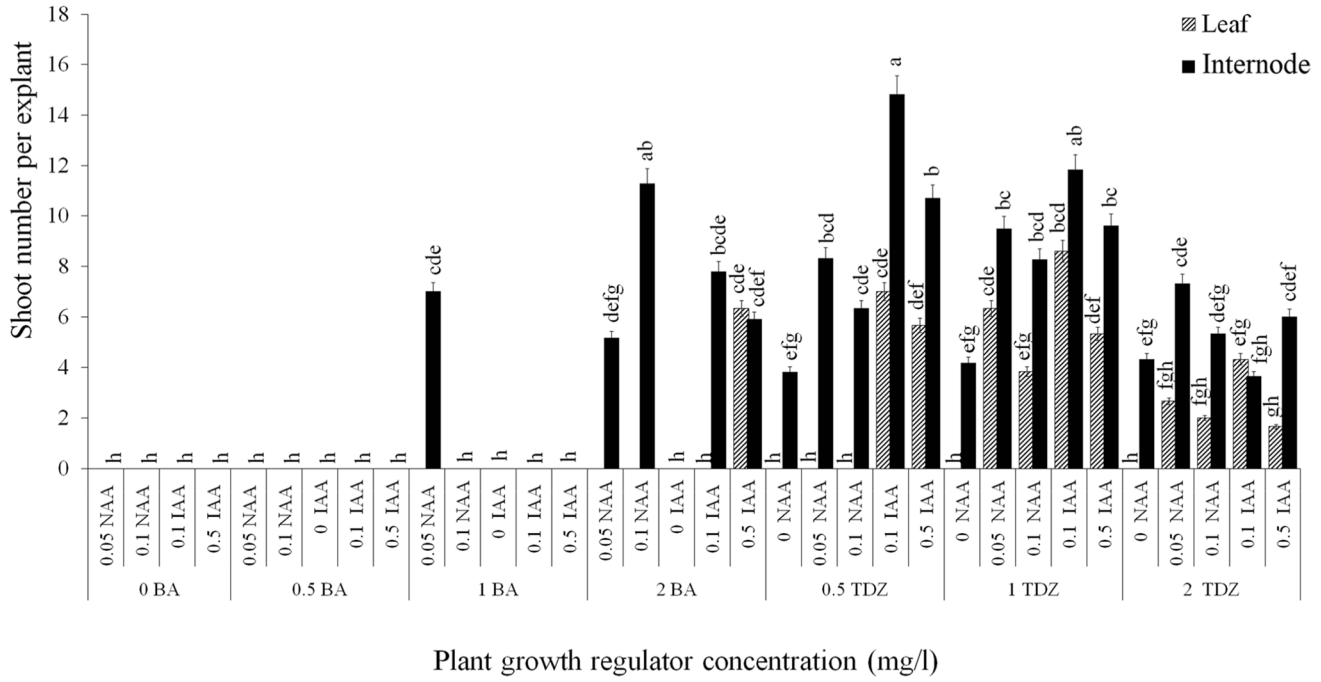

Figure 6. Effects of plant growth regulators on the average number of shoots in leaf and internode explants of *P. europaea*. The data represent the mean ± SD of three independent experiments. The means indicated by the same letters are not significantly different by Duncan's multiple range test ($p \leq .05$).

Figure 7. Direct shoot organogenesis process of *P. europaea*. (***a***–***c***) Induction of buds and shoots from internode explants on MS medium containing 0.5 mg/l TDZ and 0.1 mg/l IAA. (***d***, ***e***) Induction of buds and shoots from leaf explants on MS medium containing 0.5 mg/l TDZ and 0.1 mg/l IAA. (***f***–***h***) Transfer to the hormone-free MS medium.

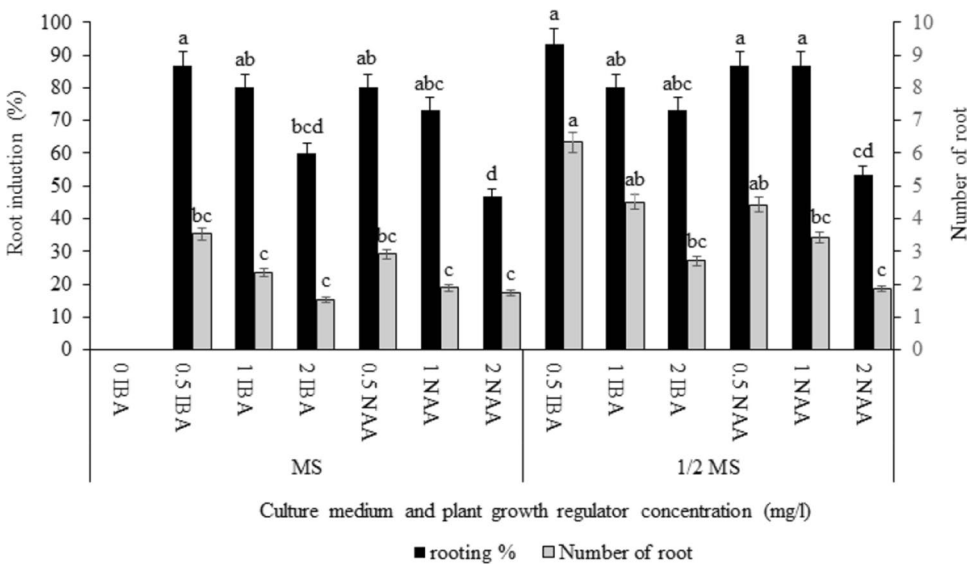

Figure 8. Effects of culture medium strength and plant growth regulators on root induction and the average number of roots. The data represent the mean ± SD of three independent experiments. The means designated by the same letters are not significantly different by Duncan's multiple range test ($p \leq .05$).

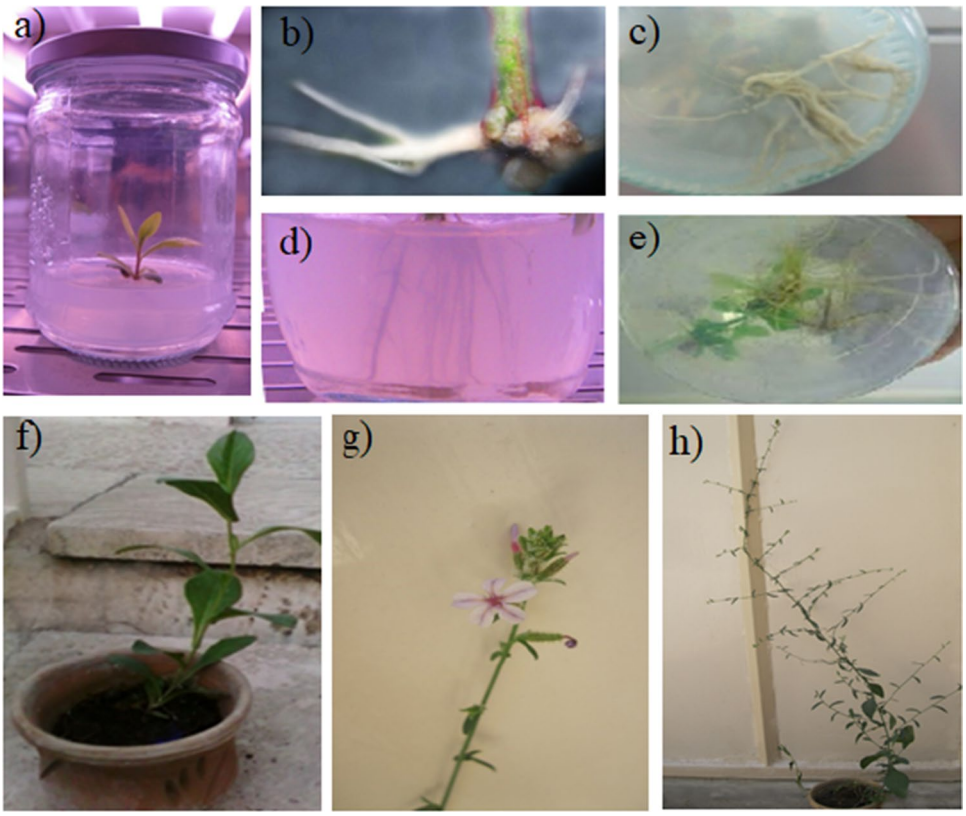

Figure 9. ***(a)*** Regenerated seedling. (***b–e) In vitro*** rooting. (***g***, ***h***) Acclimatization of *P. europaea* plantlets under *ex vitro* conditions.

the bags. After 1 mo, the hardened plantlets were field-transferred successfully with 100% survival rate (Fig. 9). For maintenance of root function in free conditions, the environment of root has been changed gradually.

Capacity of the regenerated plantlets for plumbagin biosynthesis The results of the HPLC analysis showed that the regenerated and micro-propagated plantlets were able to produce plumbagin in their roots (1.54 and 1.92 mg g^{-1} dry weight, respectively), which was considerably higher than the amount in their aerial parts (0.22 and 0.15 mg g^{-1} dry weight) (Table 1). Of note, the differences between the plumbagin quantities for same organs of regenerated and micro-propagated plantlets were not statistically significant. Similarly, the roots of wild plants contained higher plumbagin content (1.78 mg g^{-1} dry weight) than the aerial

Table 1. Plumbagin content in different studied organs of *P. europaea*. The data represent the mean ± SD of three independent experiments. The means followed by the same letters are not significantly different by Duncan's multiple range test ($p \leq .05$).

Plumbagin content (mg/g dw)	Organ
1.78 ± 0.22 a	Natural plant root
1.54 ± 0.26 a	Regenerated plant root
1.92 ± 0.17 a	Micro-propagated plant root
0.10 ± 0.01 b	Aerial parts of natural plant
0.22 ± 0.03 b	Aerial parts of regenerated plant
0.15 ± 0.02 b	Aerial parts of micro-propagated plant

parts (0.10 mg g^{-1} dry weight). Although the plumbagin content of roots in both wild and propagated plants showed no significant difference (Table 1), the time period required for the *in vitro* plant propagation was much shorter than for wild plants. Besides, using this technique, the dependence of plants to nature and the mass destruction of natural resources will be reduced. However, further research is needed to improve massive *in vitro* platelet production. In our previous works, we found high plumbagin content in hairy roots (3.3 mg g^{-1} dry weight) and in suspension cell culture (0.9 mg g^{-1} dry weight) (Beigmohamadi *et al.* 2019; 2020). Nevertheless, the advantage of the *in vitro* propagation to hairy root culture is the faster and higher biomass production (fresh weight and dry weight). Thus, the current work presenting the remarkable production of plumbagin beside the micropropagation, shoot organogenesis, and regeneration of *P. europaea* by tissue culture techniques may serve as a basis for further *in vitro* researches.

Conclusion

Our study explains the role of TDZ for shoot organogenesis of *P. europaea* which could be useful for large-scale plant multiplication and conservation. We found three key factors for enhancing successful regeneration: (1) source of explants, (2) suitable combination and concentration of growth regulators, and (3) culture conditions. The most effective methods for rapid proliferation of plants in tissue culture are direct regeneration and micropropagation, which saves time and stability of genetics and reduces the somaclonal diversity. It could be used also for genetic transformation and breeding through a biotechnological approach. Our results revealed that roots of *in vitro* propagated plants of *P. europaea* are able to produce remarkable quantities of plumbagin. Conclusively, this outcome established the potential of the regenerated plants of *P. europaea* for the large-scale commercial production of plumbagin and its derivatives using the tissue and organ cultures.

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