Research Article

# Liquid Culture System for *In Vitro* Propagation of *Physalis minima* L. a Threatened Medicinal Plant

**Muhammad Akram[1,2*], Rashid Mahmood[3], Fahim Arshad[4] and Umer Farooq Gohar[5]**

*[1]Department of Biology, Government Shalimar Graduate College, Lahore, Punjab Higher Education Department, 54000 Lahore, Pakistan; [2]Institute of Botany, University of the Punjab, Lahore-54590, Pakistan; [3]Department of Botany, Government MAO College, Lahore, Punjab Higher Education Department, 54000 Lahore, Pakistan; [4]Department of Botany, University of Okara, Okara-56300, Pakistan; [5]Institute of Industrial Biotechnology, Government College University, Lahore-54000, Pakistan.*

**Abstract** | *Physalis minima* is a promising medicinal plant of the family Solanaceae, but its survival is under threat, and limited long-term conservation approaches are available. We employed an *in vitro* plant tissue culture technique to develop a mass propagation system for the effective multiplication and conservation of *P. minima*. Juvenile leaves were cultured on MS basal medium supplemented with 0.1, 0.5, 1.0, 2.0, and 5.0 µM thidiazuron (TDZ). As a result, 100% callus induction was achieved on the medium containing 1.0 µM TDZ. Notably, calli grown on 1.0 µM TDZ exhibited greater potential for shoot regeneration and proliferation in the growth regulator-free MS liquid medium (95.25%) as compared to the solid medium (89.00%). Shoot cultures remained viable for up to 15 days without refreshing the medium and could be sustained for 12 months when sub-cultured every 15 days. Invigoration of albino shoots with fresh MS basal medium resulted in multiple green buds, which successfully transformed into 54.60 and 652.78 healthy shoots in 30 and 120 days, respectively. These shoots were rooted, acclimatized, and transferred in field conditions. The present study thus establishes a simple *in vitro* liquid culture system for the mass propagation of *P. minima*, providing a strategy for the long-term conservation of multiple endangered plant species.

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***Correspondence** | Muhammad Akram, Department of Biology, Government Shalimar Graduate College, Lahore, Punjab Higher Education Department, 54000 Lahore, Pakistan; **Email:** m.akramks@gmail.com
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## 1. Introduction

Medicinal plants are a natural source of bioactive ingredients, which are used in both curative and preventive medical therapies (Timalsina *et al.*, 2021; Kim *et al.*, 2017). More than 80% of the world population has been estimated to rely on medicinal plants or their derivatives for common healthcare needs, in complementary and traditional medicines (Mbuni *et al.*, 2020). Specifically, this is more common in third world countries (Mbuni *et al.*, 2020; Timalsina *et al.*, 2021; Umar *et al.*, 2021),

where access to traditional drugs based on modern healthcare is scarce and costly (Popovic *et al.*, 2016). The sick people typically use combinatorial approaches to gain elevated therapeutic effects from complementary and traditional drugs (Gagnier *et al.*, 2007). Moreover, the medicinal plants are consumed as an important component of daily intake, which confer functional benefits to activating biological processes (Bansal and Chhibber, 2010; Sharifi-Rad *et al.*, 2020). This has accelerated the demand for plant-derived herbal drugs, natural health products, and secondary metabolites even in the developed world. Exploitation of medicinal plants in developing traditional drugs is therefore on the rise (Sheeba *et al.*, 2010; Intzaar *et al.*, 2013).

*Physalis minima* is commonly known as wild gooseberry, is an important medicinal plant of the family Solanaceae (Sheeba *et al.*, 2010). It is a perennial herb 20–50 cm tall in size (Durga *et al.*, 2020), and is frequently found in organic matter-rich waste as well as agrarian land, distributed around the tropical and subtropical regions of Asia (Chothani and Vaghasiya, 2012). It holds strong medicinal potential, as a diuretic and laxative, and has been used as a treatment for multiple conditions including splenomegaly and ulcer of the bladder (Canh *et al.*, 2021). Crushed leaves and fruits are used to neutralize snake bites (Karthikeyani and Janardhanan, 2003), and its plant extract possess anti-inflammatory and anticancer activities (Khan *et al.*, 2009). Sadly, *P. minima* has begun to regress sharply, particularly in economically depressed regions (Intzaar *et al.*, 2013). This is mainly due to its suboptimal seed germination (Singh, 2009), the extensive use of herbicides (Vaverkova *et al.*, 1995a, b), and the lack of modern cultivation practices among rural farmers in developing countries (Ahmad and Habib, 2014). Enhancing survival of *P. minima* through creating new conservation strategies is imperative. Short-term conservation strategies such as awareness campaigns among farmers are planned, however, *in vitro* multiplication approaches are needed in the long-term, serving as a promising alternative strategy for rapid conservation of *P. minima*.

*In vitro* plant tissue culture technology is a suitable alternative to enhance the survival of medicinal plants (Afroz *et al.*, 2009; Intzaar *et al.*, 2013). It is based on seeding any tiny piece of the target plant on a defined growth medium, producing a large number of calli, which then have ample potential to be transformed into numerous highly proliferative shoots and adult plants (Jones *et al.*, 2007). A supportive growth medium is requisite for effective callus induction and requires a watchful supplementation of plant growth regulators. Thidiazuron (TDZ ) is a strong growth regulator, which has cytokinin and auxin-like properties for different morphogenic responses (Murthy *et al.*, 1998). It plays a substantial role in *in vitro* seed germination and somatic embryogenesis in multiple herbaceous and woody plants (Akram and Aftab, 2015a, b; Akram and Aftab, 2016). The concentration of TDZ is plant specific (Dewir *et al.*, 2018; Xiong *et al.*, 2022; Dalavi *et al.*, 2023), and it is therefore imperative to explore an appropriate concentration of TDZ that could support *in vitro* propagation of *P. minima* while preserving its normal growth characteristics.

Thus, objective of the present investigation was to optimize *in vitro* propagation method for mass multiplication and conservation of *P. minima* using TDZ in a liquid medium.

## 2. Materials and Methods

### *2.1 Procurement of plant material and culture conditions*

Leaves of wild *P. minima* were collected from the turmeric field crop located in village Dhing Shah Tehsil and District Kasur, Punjab, Pakistan in September. Juvenile leaves were collected from top of the plant brought to Plant Tissue Culture Lab and thoroughly washed under running tap water. Plant was dipped in 1% detergent solution (w/v) (Ahmad *et al.*, 2022) (Bright, Colgate Palmolive, Pakistan) for 10 minutes and then sanitized using 10% (v/v) Robin Bleach (Rekitts and Benkiser Group, Pakistan). Such explants were then washed with sterile water under cabinet chamber. The culture medium was autoclaved (Labtron, LVA-F22 UK) at 121 °C and 104 kilo Pascal pressure for 15 minutes and incubated at room temperature (25±2°C) under defined culture conditions for 16 hours photoperiod (35 µmole $m^{-1}s^{-1}$ provided with white fluorescent tube light, Philips). Leaves were inoculated on the sterilized culture medium.

### *2.2 Callus induction*

For callus induction, discs of 5 × 15 $mm^2$ leaf explants were cultured (abaxial side) on Murashige and Skoog (1962) (MS) basal medium. One leaf disc explant was used per culture vessel per treatment. Ten replicates/

culture vessels were used for each treatment and the experiment was repeated thrice. Solution was prepared by dissolving the salts separately, which was then fortified with 0.1, 0.5, 1.0, 2.0 or 5.0 µM Thidiazuron (TDZ) (Sigma Aldrich, USA) for callus induction. At day 14, the rate of callus induction and morphology were collected.

### *2.3 Shoot regeneration*

A one-gram callus was used per culture vessel containing MS liquid as well as agar-based (Agar, Phytotechnology Labs) solid medium devoid of any plant growth regulators (PGRs) in three tissue culture vessels including 150 mL capacity glass jars (25 mL active culture volume) (Mitchells), 25 × 150 mm culture tubes (Pyrex; 10 mL active culture volume), and 250 mL capacity Erlenmeyer flasks (50 mL active culture volume). All cultures were sub-cultured after every 15 days on the liquid and solid medium. Shoot culture from the solid medium was shifted to fresh medium after 15 days. Shoot regeneration percentage and total number of shoots per callus were recorded after 15 days of initial culture. A complete disappearance of chlorophyll in the shoot culture was regarded as an albino phenotype.

### *2.4 Regeneration of albino shoot cultures*

Clumps of albino shoots, including some basal callus portions, were inoculated in a 2-liter capacity Erlenmeyer flask containing liquid MS basal medium (150 mL active culture volume). The Erlenmeyer flask was capped with aluminum foil. The 2 Syringe Millipore filters (SMF) were adjusted in the caps of flask with one SMF for medium inlet and the 2$^{nd}$ for aeration. After every 15 days, fresh MS liquid and solid media were added to the Erlenmeyer flask. Results of shoot number per flask (10 total number of flasks) was recorded after thirty, sixty, ninety and one-twenty days of initial culture. Shoots numbers were recorded without removing the shoot cultures from the Erlenmeyer flask.

### *2.5 Root induction and hardening*

Rooting of shoots was carried out in half strength MS medium supplemented with indole-3-butyric acid (IBA) or Naphthalene Acetic Acid (NAA) (Sigma) @ 1, 2, 3 or 4 µM. Rooting percentage, number and length of roots were observed after 10 days of culture. Roots were immersed in 1% solution of Dithane fungicide (M45, Corteva) and shifted in plastic pots with 1:2 ratio of garden soil and peat moss. After 35 days, the rate of plantlet survival was recorded and transferred to the field conditions.

### *2.6 Data analysis*

A completely randomized design was applied for data collection. The data was evaluated using ANOVA and Duncan's multiple range test (DMRT) to determine statistical difference between the means of any two treatment groups at $p<0.05$. Binomial data were evaluated via logistic regression. Poisson regression was used for count data. $R^2$ where applicable was used to determine Polynomial contrast. At least ten replicate cultures per treatment group were used and every experiment was repeated thrice using SPSS v 16.0. (SAS, USA).

## 3. Results and Discussion

### *3.1 Callus induction*

Thidiazuron (TDZ) has been shown to promote efficient callus induction, therefore we first investigated effective concentrations of TDZ in Murashige and Skoog (MS) basal medium to support *P. minima* callus induction. For this, the medium was supplemented with five concentrations of TDZ (0.1, 0.5, 1.0, 2.0, 5.0 µM) and callus induction was carried out (Figure 1) from juvenile leaf explants (Figure 2A). Swelling of the explants was observed after 6 days of culture, which was followed by the initiation of callus from the cut margins of the explants across the five culture conditions (Figure 1B). The callus induction rate was measured at day 14 of the initial culture and data was evaluated using the polynomial contrast approach, which revealed a significant correlation between callus formation and TDZ 1.0 µM (Pearson coefficient of determination $R^2 = 0.8697$, $p = 0.001$). 100% callus formation was observed on medium containing 1.0 µM TDZ (Figure 1A). However, callus formation began to decline at 2.0 µM or higher TDZ (Figure 1A). It is noteworthy that the morphology of calli was different in different TDZ concentrations (Figure 1B-H). While 0.1-0.5 µM TDZ produced whitish and granular calli (Figure 1B-E), the 1.0 µM or higher concentration of TDZ resulted in nodular and morphogenic green calli (Figure 1F-H). This data showed that TDZ supplementation could result in varied morphogenic effects on *P. minima* calli, and the 1.0 µM appeared optimal for maximal callus formation in the span of 14 days.

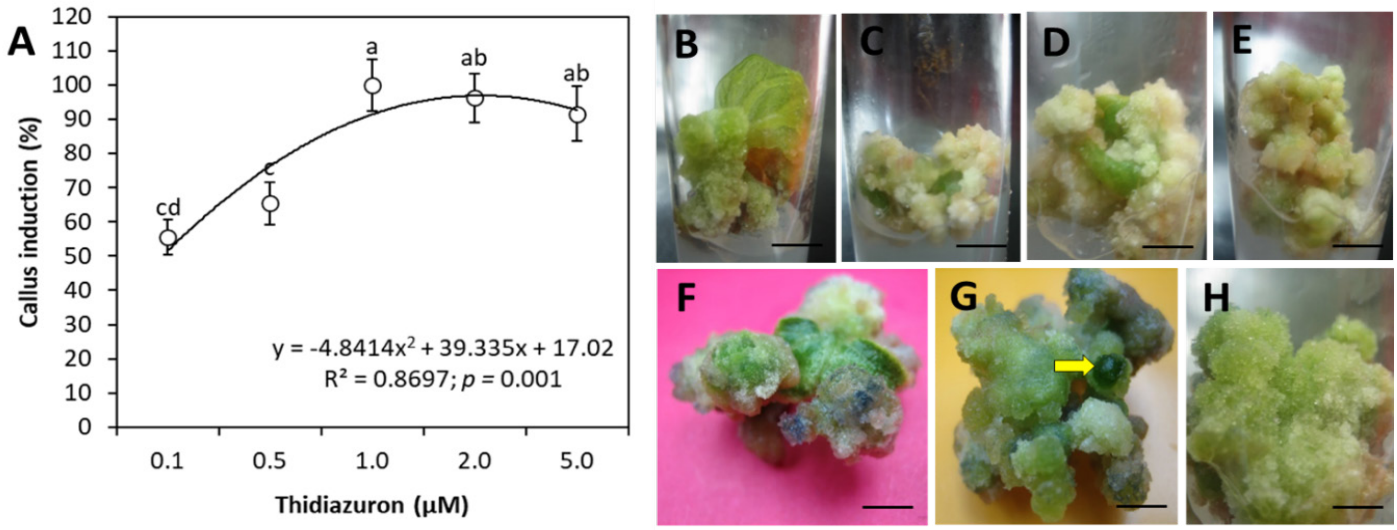


**Figure 1: Callus induction on MS medium from leaf explants of wild *P. minima*.**

(A) Vertical bars above and below the circles are ± SE of the mean. Letters in the superscript in rows represent significant difference amongst the means of the two groups evaluated by the ANOVA followed by DMR post hoc test. Y in equation indicates the values of dependent variable and $R^2$ is the Pearson coefficient of determination describing the covariance of the two variables (callus induction and TDZ concentration) divided by the product of their standard deviations. (B) Callus from cut margins of leaf explant on 0.1 µM TDZ *Bar = 11 mm*. (C, D) Off white loose callus at 0.5 µM TDZ after 10 days of culture *Bar = 10* and *11 mm*, respectively. (E) Subsequent development of callus mentioned in 'C' *Bar = 10 mm*. (F, G) Development of green nodules (yellow arrow) on green regenerating callus at 14 days of culture containing 1.0 µM TDZ *Bar = 7 mm* and *5 mm*. (H) Granular greenish callus formation in 2.0 µM TDZ medium after 10 days *Bar = 6 mm*.

### *3.2 Shoot regeneration and proliferation*

One gram of the 14-day old callus culture was transferred from the 1.0 µM TDZ concentration to the liquid or agar-based solid MS basal medium (Table 1) in three culturing vessels including Erlenmeyer flask, glass jar, and culture tube (Table 2 and 3) for shoot regeneration. Shoot regeneration initiated within 5 days of callus transfer in both liquid and solid medium in all culturing vessels (Figure 2B and 2C; representative photos). Notably, shoot regeneration rate and numbers were significantly higher in the liquid medium as compared to the solid medium at 15 days post culture transfer (99.25% with 35.00 shoot numbers versus 82.12 with 25.45; also see Table 1). The shoot regeneration appeared more pronounced in glass jars (99.25%) than the culture tubes (95.33%) and the Erlenmeyer flasks (80.23%) (Tables 1 and 3). The agar-based solid medium also supported shoot regeneration albeit with significantly lower regeneration rate and shoot numbers (Table 1). Interestingly, subculturing of the shoot culture before or at day 15 could maintain the proliferation propensity for up to 12 months with no visible morphological abnormality observed in the shoot regeneration or proliferation as demonstrated in Figure 2 C. Together, this data suggested that 1.0 µM TDZ supplementation to the MS basal medium was sufficient to prime leaf explant derived calli to display elevated potential in shoot regeneration and proliferation in basal liquid medium.

**Table 1: Shoot regeneration from callus cultures induced by 1.0 µM TDZ after 15 days of *P. minima*.**

| MS Medium | Shoot regeneration (%) | Number of shoots |
|---|---|---|
| Liquid | 99.25±6.32[a] | 35.00±4.25[a] |
| Agar | 82.12±4.25[ab] | 25.45±2.45[b] |
| Regression | p = 0.0322[1] | p = 0.0011[2] |

Data is presented as mean values (±SE). Letters in the superscript in rows represent significant difference amongst the means of the two groups evaluated by the ANOVA followed by DMRT. At least 10 replicate culture of three repetitions. [1]Logistic regression, [2]Poisson regression.

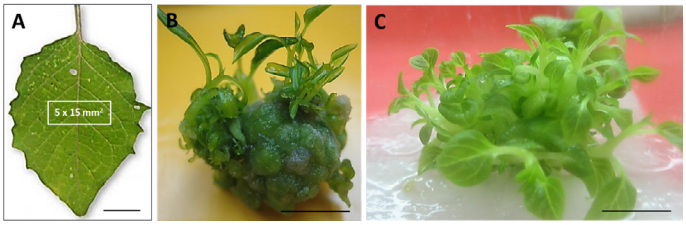


**Figure 2: Shoot organogenesis from leaf of wild *P. minima*.**

(A) Leaf explant *Bar=10 mm*. (B) Five-day old shoots formed from callus tissue previously cultured on TDZ (1.0 µM) *Bar=7 mm*. (C). After 15 days on the same medium shoots were further proliferated. *Bar = 5 mm*.

### *3.3 High frequency shoot formation*

The propensity of shoot regeneration and proliferation

**Table 2: *P. minima* shoot multiplication from the albino shoots in the basal liquid culture.**

| MS basal medium | Number of multiple shoot formation in Erlenmeyer Flasks | | | |
|---|---|---|---|---|
| | 30 days | 60 days | 90 days | 120 days |
| Liquid medium | 54.60±11.32$^{d}$ | 138.75±14.36$^{c}$ | 345.32±62.35$^{b}$ | 652.78±25.66$^{a}$ |
| Solid medium | 34.12±14.25$^{d}$ | 145.32±32.25$^{c}$ | 241.02±27.32$^{ab}$ | 352.78±45.23$^{a}$ |

Data is presented as mean values (±SE). Letter in the superscript in rows represent significant difference amongst the means of the two groups evaluated by the ANOVA followed by DMRT. At least 10 replicates with 3 repetitions were carried out.

**Table 3: Shoot regeneration from callus cultures induced by 1.0 µM TDZ after 15 days of *P. minima*.**

| MS medium | Erlenmeyer flasks | | Culture tubes | |
|---|---|---|---|---|
| | Shoot regeneration (%) | Number of shoots | Shoot regeneration (%) | Number of shoots |
| Liquid | 80.23±5.23$^{a}$ | 19.12±3.25$^{a}$ | 95.33±5.78$^{a}$ | 18.25±4.25$^{a}$ |
| Agar | 75.75±4.12$^{b}$ | 16.23±4.25$^{ab}$ | 76.52±6.32$^{b}$ | 14.33±5.65$^{ab}$ |
| Regression | p=0.0221$^{1}$ | p=0.0001$^{2}$ | p=0.0330$^{1}$ | p=0.0021$^{2}$ |

Mean values (±SE) followed by different letters are significantly different according to Duncan's multiple range test p<0.05. Each value is the mean of 10 replicates and the experiment was repeated thrice. $^{1}$Logistic regression, $^{2}$Poisson regression.

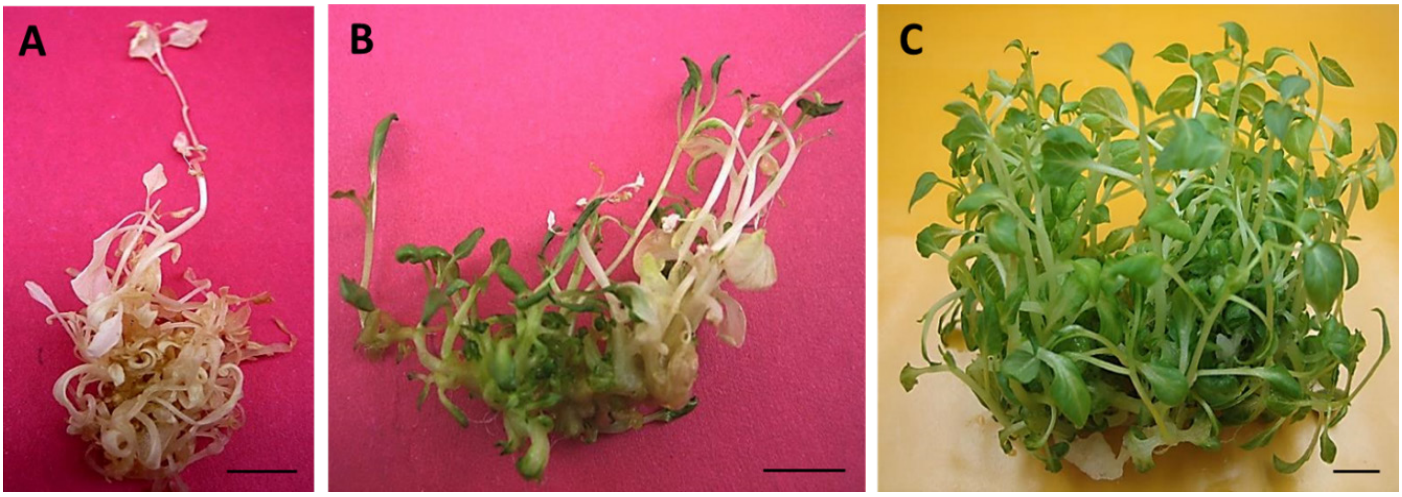


**Figure 3: Regeneration potential of the albino shoots derived from the 1.0 µM TDZ primed calli.**
(A) A representative photograph displaying emergence of the albino shoot phenotype when medium was not refreshed within 15 days. (B) A representative photograph of re-greening albino shoots with invigoration with fresh MS medium for 10 days. (C) A representative photograph of the albino shoot culture displaying high frequency of shoot multiplication on the MS liquid medium. *Bar = 15 mm.*

remained stable for 15 days without refreshing the medium, but beyond this, the albinism prevailed with shoots displaying visible chlorophyll loss (Figure 3A). We then tested whether the albino shoots derived from the 1.0 µM TDZ-primed calli could be rejuvenated to high frequency shoot formation. For this, 20-days old albino shoots were invigorated in fresh MS liquid medium in an Erlenmeyer flask, with solid medium used as a control. Intriguingly, multiple shoot buds appeared as early as 10 days of invigoration, which transformed into lush green healthy shoots (Figure 3B). The liquid medium produced significantly more green shoots (54.60) in the albino shoot culture than the solid medium (34.12) at 30 days of rejuvenation. Moreover, the number of shoots continued to increase over time and reached 138.75, 345.32 and 652.78 at 60, 90, and 120-days of rejuvenation, respectively (Table 2). It is noteworthy that a similar increasing trend of shoot formation and multiplication over time was observed in the albino shoots cultured on the solid medium (Table 2). This further pointed to the existence of elevated shoot regenerative potential imparted by the 1.0 µM TDZ priming during callus induction. We also tested albino shoot regeneration in the glass jar and culture tube culturing vessels (Table 2). However, shoot formation frequency was high in only the Erlenmeyer flask, possibly due to adequate nutrient diffusion and aeration in the spacious vessel (Table 2). Overall, shoot formation and multiplication remained visibly normal throughout experiments (Figure 3C), however, some minor signs of hyperhydricity were noted in a few cultures grown in the liquid medium (35.33%) (Table 4).

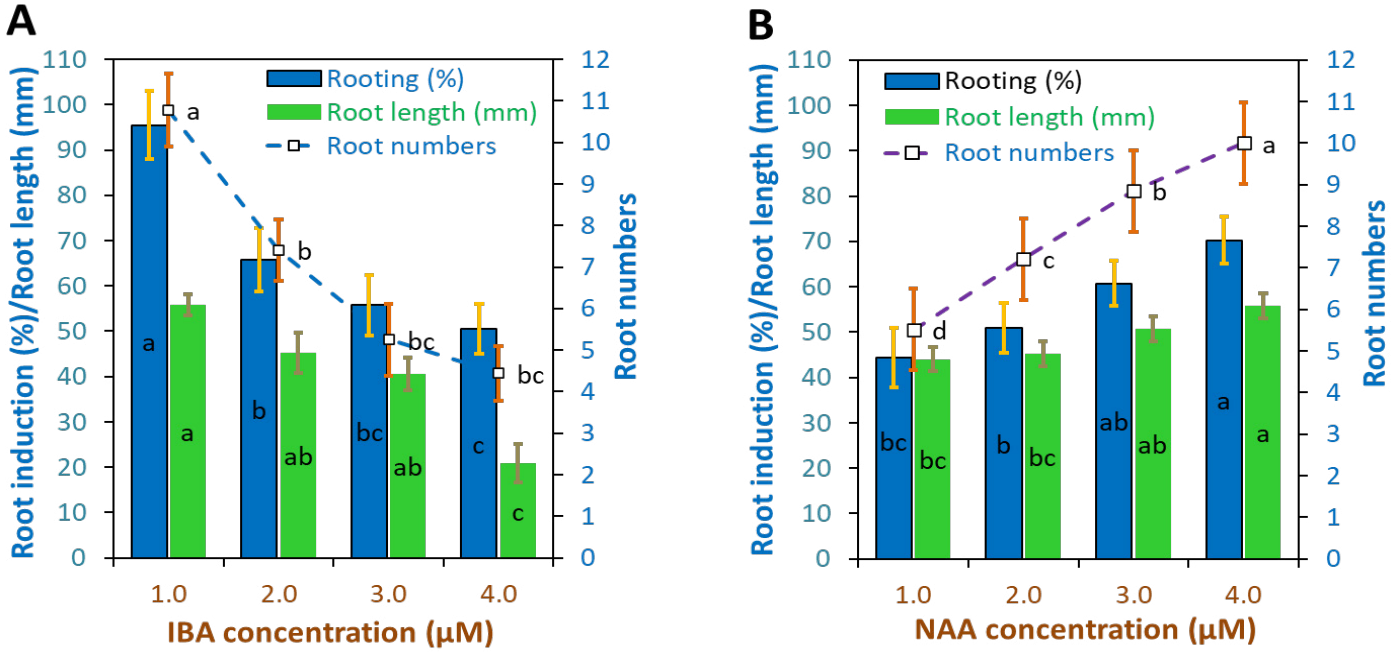


**Figure 4: *In vitro* rooting and survival of *P. minima* plantlets in half strength MS medium.**
(A) Percentage of rooting, root length and root number obtained with 1, 2, 3, and 4 µM IBA. (B) Percentage of rooting, root length in mm and root numbers with NAA @ 1, 2, 3, and 4 µM after 10 days of culture. Bar charts represent the means and the vertical bars over bar charts represent ±SE.

**Table 4: Shoot hyperhydricity prevalence in different culture media the Erlenmeyer flask. The culture was revised from the albino shoot of *P. minima*.**

| MS medium | *Hyperhydricity (%) |
|---|---|
| Liquid | 35.33 ± 4.36 |
| Agar | 25.36 ± 3.25 |

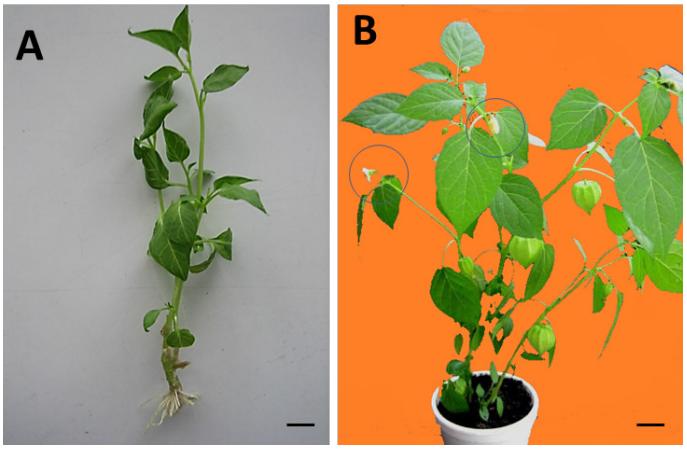


**Figure 5: Plantlet showing roots and fully acclimatized plants in ½ MS medium.**
(A) Representative photographs of a proliferating shoot displaying successful rooting with IBA @ 1.0 µM. *Bar = 15 mm*. (B) Representative pool of the 35-days old adult *P. minima* plant propagated via currently established protocol. The plants successfully produced flowers and fruits (circles). *Bar = 15 mm.*

### *3.4 Root induction and hardening*

The proliferating shoots (Figure 2 and 3) derived from the 1.0 µM TDZ-primed callus (Figure 1) were rooted in half strength MS medium + 1.0, 2.0, 3.0 or 4.0 µM either IBA or NAA. The rate of rooting was highest (95.45%) with 10.78 number of roots and 55.85 mm root length was observed in medium containing 1.0 µM IBA (Figure 4A) as compared to the medium containing the same concentration of NAA, which provided 70.21% root induction with 10 mean number of shoots and 55.74 mm root length after 35 days (Figure 4B). Plantlets acquiring 95% roots (Figure 5A) were shifted to the glasshouse for acclimatization. They were then further planted in field conditions. The fully acclimatized adult plants started to produce flowers and fruits successfully at the age of 35 days (Figure 5B).

Medicinal plants play a crucial role in socio-cultural, healthcare, and pharmaceutical industries all over the world (Mbuni *et al.*, 2020). Poor seed germination (Singh, 2009), massive use of herbicides (Vaverkova *et al.*, 1995a, b), and over-harvesting of fields by farmers have caused significant threat to the existence of medicinal herbaceous plants, including *P. minima* (wild gooseberry). Towards addressing this problem, the present study has developed a straightforward *in vitro* tissue culture approach as a long-term conservation strategy to preserve wild gooseberry plant from going extinct (Canh *et al.*, 2021; Wu *et al.*, 2020). This strategy is based on efficient callus

induction mediated by a low concentration of thidiazuron (TDZ; 1.0 µM) in basal MS medium. This initial TDZ-priming appears essential as it promotes effective shoot multiplication of *P. minima* in the plant growth regulator-free basal medium (Anjani and Kumar, 2018). However, the underlying mechanism as to how TDZ-mediated callus induction exhibits high potential for shoot regeneration and proliferation remains elusive. Thidiazuron is reported to be stable (Mok *et al.*, 1982), and its metabolism is extremely slow in plant tissue culture (Mok *et al.*, 1985). Additionally, it is likely that the remnants of TDZ-derived metabolites play a supporting role (Mok *et al.*, 1985). Indeed, two new TDZ derivatives (1-[1, 2, 3] thiadiazol-5-yl-3-(3-trifluoromethoxy-phenyl) urea (3FMTDZ) and 1-[2-(2-hydroxyethyl) phenyl]-3-(1, 2, 3-thiadiazol-5-yl) urea) have been identified as being very potent inhibitors of cytokinin oxidase/dehydrogenase (Nisler *et al.*, 2016) accumulate in plant tissues via suppressing the activity of cytokinin oxidase (Horgan *et al.*, 1988; Hare *et al.*, 1994), and accumulated cytokinins can in return promote shoot multiplication. Also, TDZ modulation of gibberellin biosynthesis has been proposed too (Ali *et al.*, 2022). However, unbiased metabolomic investigations as done before (Erland *et al.*, 2020) focusing on different stages of micropropagations of *P. minima* are warranted for further clarification in future studies.

Previous efforts in this field have focused on propagating *P. minima* through artificial means, which includes *in vitro* synthetic seed production and growth induction by low temperature and osmotic agents of sucrose, mannitol, and sorbitol in tissue culture medium (Yücesan *et al.*, 2015; Nasiruddin and Islam, 2018; Rezende *et al.*, 2018; Yadav *et al.*, 2019). While application of such methodologies could serve the short-term conservation needs of *P. minima*, the present method ensures long-term culture survival for up to 12 months by refreshing medium within 15 days, thus providing the foundation for a long-term conservation strategy for *P. minima* (Figure 3C).

Rapid shoot regeneration has a direct relationship with shoot organogenesis, which has been reported previously to respond variably to liquid and solid media (Jones *et al.*, 2007; Afroz *et al.*, 2009; Intzaar *et al.*, 2013). We and others have investigated multiple aspects of shoot organogenesis in liquid and solid media, with the shoots of woody (Akram and Aftab, 2016) and medicinal (Hailu *et al.*, 2022) plants tending to grow longer and quicker in liquid medium. The abundant shoot formation of *P. minima* in the present study is therefore consistent with previous reports describing the efficacious role of liquid medium (Akram and Aftab, 2016; Hailu *et al.*, 2022). Moreover, shoot regeneration is more pronounced in the Erlenmeyer flask, which is also in line with previous reports describing how culturing vessels with larger volume are effective for fast shoot formation in liquid medium (Kaçar *et al.*, 2010). This could also be due to more extensive physical contact between medium and shoots, which may enhance nutrient uptake and gaseous exchange between medium and shoot biomass (Choi *et al.*, 2001).

We noticed that the shoots derived from the TDZ-primed calli begin to turn albino when medium was not refreshed at day 15 of shoot regeneration. Albinism often reflects differences in genotype, environmental conditions, or genome-based modifications that are reported to be associated with TDZ treatment (Kumari *et al.*, 2009; Lin and Chang, 1998). Interestingly, the shoot albinism appearing in the present study does not seem to be associated with TDZ treatment, as re-greening of albino shoots was promptly achieved by invigorating them in fresh basal medium. This suggests that the prevalence of the albinism may not be a consequence of TDZ treatment. Rather, it could well be associated with nutrient depletion in the culture medium and can swiftly be reversed upon supplying nutrient-rich medium. The reason behind this complete reversal of shoot albinism in *P. minima* remains puzzling and will require more investigation in future endeavors. However, it is possible that culture refreshed with sucrose could switch on some chlorophyll synthesis pathways resulting in rejuvenation of albino shoots and abundant shoot formation (Wolff and Price, 1960). Alternatively, TDZ-priming may induce a shift in metabolic reprograming in the calli leading to reduced requirement for nutrients, and a survival of shoot culture even in the absence of chlorophyl. This is consistent with the notion that TDZ treatment could result in the accelerated transcription, *in vitro,* of systems containing chromatin and RNA polymerase I from TDZ-treated leaves (Karavaiko *et al.*, 2004). Realization of such a shift in metabolic reprogramming in *in vitro* propagation of *P. minima* is highly speculative and deserves further investigation at the genomic level.

The present study encountered two challenges; firstly, marginal contamination events were observed during initial commencement of experiments including callus induction. This issue was curtailed via improving and strictly adhering to the recommended aseptic guidelines for plant tissues (Singh, 2018; Durul and Memis, 2022). Overall, 90% of our culture remained free from any contamination (Table 3). Secondly, like others (Kevers *et al*., 2004; Martinez and Deklerk, 2010), this study also encountered the hyperhydricity challenge, which was confined to only shoots growing in liquid medium. This phenomenon was pronounced when the cultivation time exceeded 120 days (35%; Table 4). Future endeavors will be made to reverse the hyperhydricity issue by optimizing liquid medium components, possibly through controlled supplementation of cobalt and silver salts. Recently, addition of a combination of $CoCl_2$ and $AgNO_3$ salts has been shown to completely inhibit hyperhydricity without compromising shoot numbers (Sreelekshmi and Siril, 2021). Additionally, beneficial effects of custom-made culturing vessels with elevated aeration via agitation could be explored, as proper aeration of the culture medium is negatively corelated with hyperhydricity appearance (Reyes-Vera *et al*., 2008).

In conclusion, the present study demonstrated 100% callus induction from juvenile leaves explants of *P. minima* in a MS basal medium containing 1.0 µM TDZ. Importantly, TDZ-primed calli exhibited ample potential for efficient shoot regeneration and proliferation in the MS liquid medium devoid of any plant growth regulators. Moreover, emerging shoots from TDZ-primed calli are lush green, flourishing promptly in spacious Erlenmeyer culturing flasks. These shoots were effectively rooted in ½ MS basal medium supplemented with 1.0 µM IBA. The plantlets were then able to be acclimatized and transferred to the field successfully. In summary, the present protocol provides a simple and economical *in vitro* liquid culture system for massive propagation of *P. minima*, thus setting the stage for developing a long-term conservation strategy for *P. minima* that could be employed for the other multiple endangered species of medicinal plants.

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## Novelty Statement

Albino shoots reverted back to green shoot clusters after invigorating with fresh medium.

## Author's Contribution

Muhammad Akram; experimental work and write-up.
Rashid Mahmood; editing and proof reading.
Fahim Arshad; statistical analysis.
Umer Farooq Hohar; Technical assistance.

*Conflicts of interest*

The authors have declared no conflict of interest.

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