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ORIGINAL ARTICLE

# In vitro propagation of *Fritillaria pontica* Wahlenb.

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## Abstract

The present research aimed to develop an in vitro propagation protocol for *Fritillaria pontica*, a geophyte naturally distributed in Southeastern Europe and Northwestern Türkiye, by testing different temperature regimes and exogenous plant growth regulator treatments (PGR). Thidiazuron (TDZ) treatment at 2.0 mg $L^{-1}$, applied during a repeated cycle of short-term cold treatment (RSCT), induced the highest seed germination rate (60%) and shoot production (0.6 shoot per seed). Root formation was minimal under PGR treatments, regardless of the incubation temperature regime. 6-benzylaminopurine (BAP) at 2.0 mg $L^{-1}$ induced the highest bulblet production per seed (0.26 bulblet per seed) and most elongated shoots (1.46 cm per seed) treated with prolonged-term cold (PCT). The calli around the seedlings were cultured on the same medium enriched with kinetin (KIN) for organ induction for 2 and 6 months. Control medium induced the highest caulogenesis response at the end of both periods. Bulblets were also treated with TDZ, indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA) for the same periods as calli. The control medium yielded the highest shoot and root production from bulblet cultures after 2 months. TDZ and NAA at 0.5 mg $L^{-1}$ yielded the highest shoot and root production, respectively, after 6 months. The shoots derived through indirect organogenesis responded to IBA treatments only during a 6-month incubation. IBA at 2.0 mg $L^{-1}$ yielded the highest rhizogenic response (3.13 roots per shoot at 1.51 cm). The methodology developed in our study would contribute to conservation studies on the Fritillaria species.

## Key message

A gradually increasing temperature regime and thidiazuron at a higher concentration increase germination and organ development, while certain kinetin and indole-3-butyric acid treatments trigger callogenesis or rooting in *Fritillaria pontica*.

**Keywords** Auxin · Cytokinin · Micropropagation · Organogenesis · Pontic fritillary · Rare species

## Introduction

Most of the Liliaceae (lily) family consists of bulbous plants (Rønsted et al. 2005). The genus Fritillaria L. occurs in three biodiversity hotspots: Iran-Anatolia, the Mediterranean forest region, and the Horn of Africa. This genus is found in the Middle East and Western Asia and comprises approximately 170 taxa (Advay et al. 2022; Rix 2019). Therefore, Türkiye is considered one of the centers of genetic diversity for the genus (Eker and Tekşen 2023). *Fritillaria pontica* (Pontic fritillary) is a herbaceous, perennial, bulbous geophyte plant species that typically grows to 15–45 cm in height and has aesthetic bell-shaped flowers, which bloom between March and April (POWO 2025; RHS 2025). The species is also considered a shade-tolerant ornamental plant valued for its medicinal properties and horticultural appeal. The species grows naturally in the Balkans and Northern Türkiye (Teofilova and Naumova 2020), and its habitat includes wooded areas, roadsides, forest clearings, and scrublands. However, this species faces several risks in the areas where it is found. The primary threat to *F. pontica* is habitat loss and unintentional human collecting, which have

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raised concerns about its potential extinction in the wild. In addition to these challenges, Teofilova and Naumova (2020) reported that the leaf-eating insect *Lilioceris faldermanni* in Bulgaria has a new host plant, *F. pontica*. They emphasised that this insect poses a real threat to *F. pontica*, which is classified as LC (Least Concern) by the International Union for Conservation of Nature (IUCN; Bazos 2018).

Fritillaria species are commonly cultivated as ornamental garden plants, and the dried bulbs of some species are used in traditional medicine in Türkiye (Karakaş Metin et al. 2013), Japan (Rashid and Yaqoob 2021), and China (Qu et al. 2022). These bulbs serve as raw materials to relieve pain, reduce phlegm, lower fever, alleviate coughing, and promote detoxification, and contain specific alkaloids that have attracted the pharmaceutical industry's interest due to these properties (Wang et al. 2016, 2022). Beyond their medicinal uses, *F. pontica* also holds significant economic value due to its chemical compounds. For example, Orhan et al. (2009) determined that ethanol extracts of the species' fruits exhibit high antioxidant activity and contain small amounts of rutin and hyperoside. Furthermore, in a study examining the antibacterial and antitumor activities of plants grown in Türkiye, *F. pontica* extracts showed moderate antitumor activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Usta et al. 2014).

Seed germination studies of the Fritillaria genus remain limited in number due to its unique characteristics, such as strong dormancy-related recalcitrancy of its seeds. Conventional methods of producing mature bulbs from seeds for nearly all species in the Fritillaria genus are very slow and take five to six years (Paek and Murthy 2002). The seed testa of the species is not thick enough to limit water uptake, suggesting that epigenetic and/or hormonal mechanisms, rather than mechanical factors, underlie the species' seed dormancy. In this context, in vitro cultures are advantageous for studying dormancy-breaking treatments while providing a controlled environment for seed germination. Plant tissue culture techniques enable the rapid production of target plant species. Furthermore, as it is conducted independently of external environmental conditions, it makes a significant contribution to the conservation and propagation of these endangered species. According to the literature, most tissue culture studies on the genus Fritillaria have used bulb explants. Seed propagation studies have been conducted only with the species *F. persica* and *F. imperialis* (Kizil and Khawar 2014; Çakmak et al. 2016; Akyüz 2018). To the best of our knowledge, no tissue culture studies have been conducted on *F. pontica* yet. Therefore, the objective of this study is to develop and examine an in vitro propagation protocol for *F. pontica*, focusing on overcoming seed dormancy through dormancy-breaking methods and evaluating seed germination, to support biodiversity conservation efforts for this species.

## Materials and methods

### Seeds' collection and assessment of seed viability

Mature seeds of *Fritillaria pontica* Wahlenb. were collected in the summer of 2022 from wild populations at the Çınarlıdere Recreation Area (Derince, Kocaeli, Türkiye). The collection was conducted after obtaining permits from the General Directorate of Nature Conservation and National Parks of the Ministry of Agriculture and Forestry of Türkiye. The seeds were stored in the dark at 4 °C for 1 week until use.

Seed viability was assessed using the 2,3,5-triphenyltetrazolium chloride (TTZ) assay, following the protocol from Acemi (2020) with modifications. Seeds were immersed in distilled water for 24 h to soften the coat, then incubated in the dark at room temperature for 24 h in 1% (*w: v*) TTZ solution. After incubation, seeds were inspected with a stereomicroscope. Seeds showing pink or red were labeled viable; unstained seeds were non-viable.

### Preparation of culture medium and disinfection of seeds

A full-strength Murashige and Skoog (MS) medium with vitamins was used for all experiments (Murashige and Skoog 1962). The medium was supplemented with 30 g $L^{-1}$ sucrose and solidified using 7 g $L^{-1}$ plant agar (Duchefa Biochemie). To prevent microbial contamination, 1.0 mL $L^{-1}$ plant preservative mixture (PPM™; Plant Cell Technology) was added to all media. The pH was adjusted to 5.7–5.8 before autoclaving at 121 °C and 118 kPa for 20 min.

The seeds were treated with 70% (*v: v*) ethanol for 1 min, followed by 1% sodium hypochlorite (*v: v*) and 1–2 drops of Tween® 20 for 15 min, and rinsed three times in sterile distilled water. Following the disinfection, seeds were aseptically transferred onto sterile filter paper for desiccation prior to inoculation onto culture media.

### Seed sowing, treatments for breaking dormancy, and culture conditions

The disinfected seeds were placed onto the culture medium supplemented with thidiazuron (TDZ; Duchefa Biochemie), kinetin (KIN; Sigma-Aldrich), 6-benzylaminopurine (BAP; Caisson Labs), indole-3-butyric acid (IBA; Sigma-Aldrich), 3-indoleacetic acid (IAA; Sigma-Aldrich), or 1-naphthaleneacetic acid (NAA; Sigma-Aldrich) at concentrations of

0.25, 0.5, 1.0, and 2.0 mg $L^{-1}$ to test the effects of plant growth regulators on breaking dormancy. In the control medium, no PGRs were added. These treatments were tested for four different incubation periods. To study the effects of multiple temperature regimes across different incubation periods, the cultures were subjected to a short cold treatment at 4 °C, followed by a gradual increase to 16 °C and 23 °C. The individual treatment periods ranged from 1 to 3 months. In the control group for temperature regimes, the cultures were incubated directly at 23 °C for 3 months. Table 1 shows the treatment temperatures and their periods. The cultures maintained at 4 °C and 16 °C were kept in the dark, whereas those maintained at 23 °C were exposed to 60 µmol $m^{-2}$ $s^{-1}$ cool-white fluorescent light under a 16/8 h light/dark photoperiod.

## Effects of plant growth regulators on organogenesis

Bulblets without roots developed on the seedlings were excised and transferred to the MS medium containing TDZ, IBA, and NAA at 0.5 and 1.0 mg $L^{-1}$ to test the effects of these plant growth regulators (PGRs) on root and leaf formation from bulblets. Callus tissues that formed spontaneously around the seedlings during germination were cut into ~0.5 × 0.5 cm dimensions and subcultured in MS medium supplemented with KIN at 2.0 and 4.0 mg $L^{-1}$ to induce indirect organ development. To enhance root development, shoots developed through indirect organogenesis were cultured for 2 and 6 months in MS medium containing IBA at 0.5, 1.0, and 2.0 mg $L^{-1}$. The cultures were maintained at 23 °C for 2 and 6 months to evaluate their organogenic potential.

## Acclimatization of plantlets and genetic stability analysis

All plantlets with well-developed organs had their vessel lids replaced with filter caps for one week. At the end of this period, the plants with well-developed organs were removed from the culture vessels, and agar residues were cleaned. They were then transferred to cups containing a mixture of sterile cocopeat and perlite (*3:1*, *v: v*). To maintain moisture, the cups were covered with plastic bags for one month. The seedlings were grown in a growth chamber illuminated by full-spectrum light-emitting diodes at 25 °C and 80 µmol $m^{-2}$ $s^{-1}$.

Genomic DNA was extracted from the mother plant and two randomly selected in vitro-propagated plants using the cetyltrimethylammonium bromide (CTAB) method (Redhwan et al. 2023). The concentration and purity of the isolated DNA were verified using a spectrophotometer (BMG LabTech, SPECTROstar Nano). Seven ISSR primers (UBC-857, 859, 866, 896, 897, 898, and 899) were tested for the amplification of genetic material. For PCR amplification, each reaction was completed with 12.5 µL of Taq 2× Master mix, 1 µL of ISSR primer, 1 µL of DNA (50 ng), and sterile distilled water to a total volume of 25 µL. The PCR reaction was performed in a thermal cycler (Applied Biosystems, SimpliAmp) using the following PCR program: 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 50–53 °C for 30 s, and extension at 72 °C for 2 min; followed by a final extension at 72 °C for 10 min.

Amplified PCR products were separated by electrophoresis on a 1% (*w: v*) agarose gel prepared in 1× TAE buffer and stained with ethidium bromide. A 100 bp DNA ladder (GeneOn) was used as the molecular size standard. Electrophoresis was performed at 120 V for 60 min, and the resulting band patterns were visualized under a UV illuminator (Vilber Lourmat, ECX-F20) and scored manually. Figure 1 presents a graphical flowchart that summarizes the treatments and phases included in the study.

## Statistical analysis and data visualization

Seed viability experiments were conducted with 15 seeds per replicate. Each culture vessel contained five seeds, bulb explants, or calli and served as a replicate. Every treatment was repeated three times. Duncan's Multiple Range Test was run after one-way analysis of variance at $p < 0.05$ significance level. IBM SPSS Statistics 22 software was used for the analyses. The data were given as mean ± standard error.

The seeds that produced a plumule and a radicle were considered germinated. Germination percentage, shoot and root numbers and lengths, callus induction and growth, leaf numbers, and organ formation percentages were evaluated at the end of the incubation periods.

The developmental data were first normalized to the [0, 1] interval to perform hierarchical cluster analysis (HCA) using a heat map in the Interactive Clustered Heat Map Builder (Ryan et al. 2020). The analysis was based on

**Table 1** Temperature regimes and incubation periods tested for breaking seed dormancy in *Fritillaria pontica*

| Treatment\Temperature & period | 4 °C (months) | 16 °C (months) | 23 °C (months) | Total duration (months) |
|---|---|---|---|---|
| Control | 0 | 0 | 3 | 3 |
| Short-term cold treatment (SCT) | 1 | 1 | 1 | 3 |
| Repeated cycle of short-term cold treatment (RSCT) | 2 (1 + 1) | 2 (1 + 1) | 2 (1 + 1) | 6 |
| Intermediate-term cold treatment (ICT) | 2 | 1 | 1 | 4 |
| Prolonged-term cold treatment (PCT) | 3 | 1 | 1 | 5 |

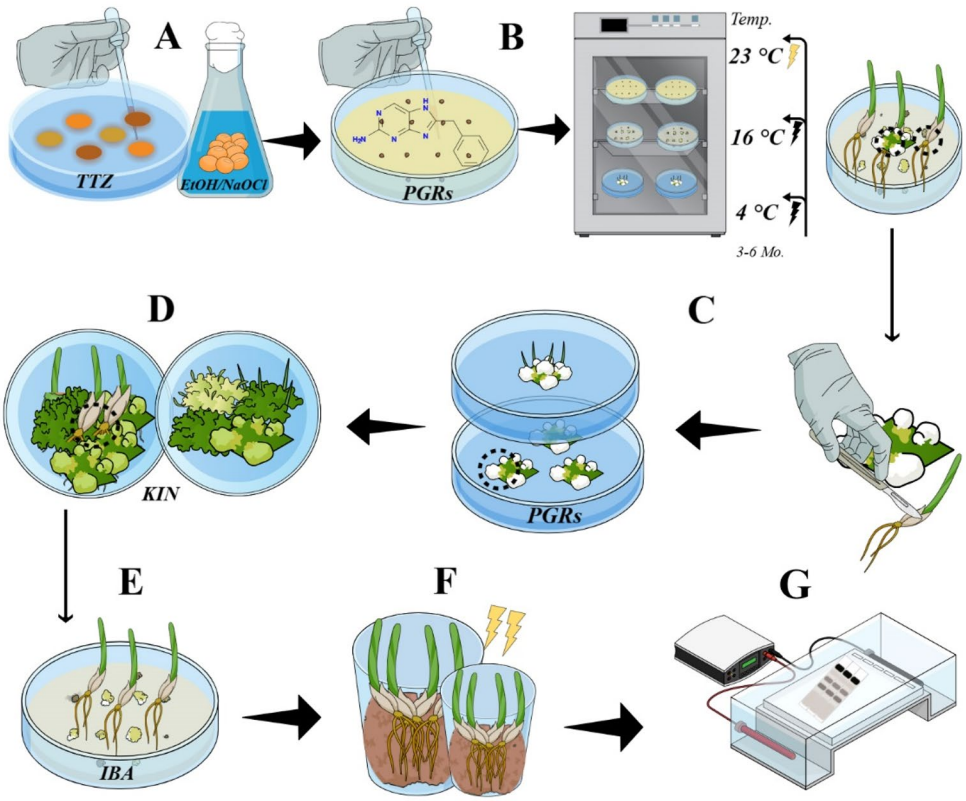



**Fig. 1** Graphical flowchart summarizing the treatments and phases involved in the in vitro propagation of *Fritillaria pontica*: seed viability assessment and disinfection (**A**), evaluation of plant growth regulator (PGR) treatments on seed germination and seedling growth under varying temperature regimes (**B**), culture of calli and bulbs on PGR-supplemented medium (**C**), assessment of kinetin (KIN)-supplemented medium on organ formation (**D**), evaluation of indole-3-butyric acid (IBA)-supplemented medium on rhizogenesis (**E**), acclimatization procedures (**F**), and analysis of genetic fidelity in selected regenerants (**G**). Figure created in the Mind the Graph platform (www.mindthegraph.com)

Euclidean distance and the complete-linkage clustering method.

## Results

### Effects of PGRs, temperature, and incubation period on seed germination

According to the TTZ seed viability test results, all seeds tested were viable (Fig. 2A). The results showed that the germination rate of seeds incubated at SCT was minimal. Interestingly, regardless of PGR treatment, when these seeds were later subjected to RSCT, germination rates increased and were the highest among all groups. Seeds incubated at ICT showed higher germination rates than those incubated at SCT. Additionally, seeds incubated at PCT showed higher germination rates than those incubated for SCT or ICT (Table 2). Among the tested PGRs, TDZ at 2.0 mg $L^{-1}$ and IBA at 1.0 and 2.0 mg $L^{-1}$ emerged as the most effective treatments for germination at the end of RSCT. These treatments were statistically similar. Seeds exposed to PCT yielded germination rates up to 26.7% when treated with 2.0 mg $L^{-1}$ BAP. However, the shorter incubation period at 4 °C (SCT) was less effective in promoting germination. The control group failed to germinate under all conditions except RSCT (Table 2). The incubation periods across different temperature regimes tested for seed germination in the presence of PGRs are summarized in Fig. 3.

### Effects of PGRs, temperature, and incubation period on seedling development

The results showed that PGR concentrations and cold incubation periods significantly affected organ development. Seeds incubated at SCT produced the fewest shoots. Seeds incubated at RSCT exhibited the highest seedling development. The highest shoot production was obtained from the medium with 2.0 mg $L^{-1}$ TDZ and 1.0–2.0 mg $L^{-1}$ IBA (Table 3). No statistically significant difference was found among these treatments. Similarly, in terms of shoot length, seeds cultured at TDZ 2.0 mg $L^{-1}$ and IBA 0.5-1.0 mg $L^{-1}$ produced the longest shoots, without any statistically significant difference (Table 4). For seeds incubated at ICT, the highest shoot production and the longest shoots were obtained in media containing NAA at 2.0 mg $L^{-1}$. After 3 months of incubation at 4 °C (PCT), the highest shoot production was recorded from the medium containing BAP at 2.0 mg $L^{-1}$ and TDZ at 1.0 or 2.0 mg $L^{-1}$. In the same trial, BAP treatment at 2.0 mg $L^{-1}$ induced the longest shoots (Fig. 2B).

Root development was very weak in all seedlings, regardless of incubation period or PGR treatment. Only the seeds subjected to RSCT and ICT treatments showed

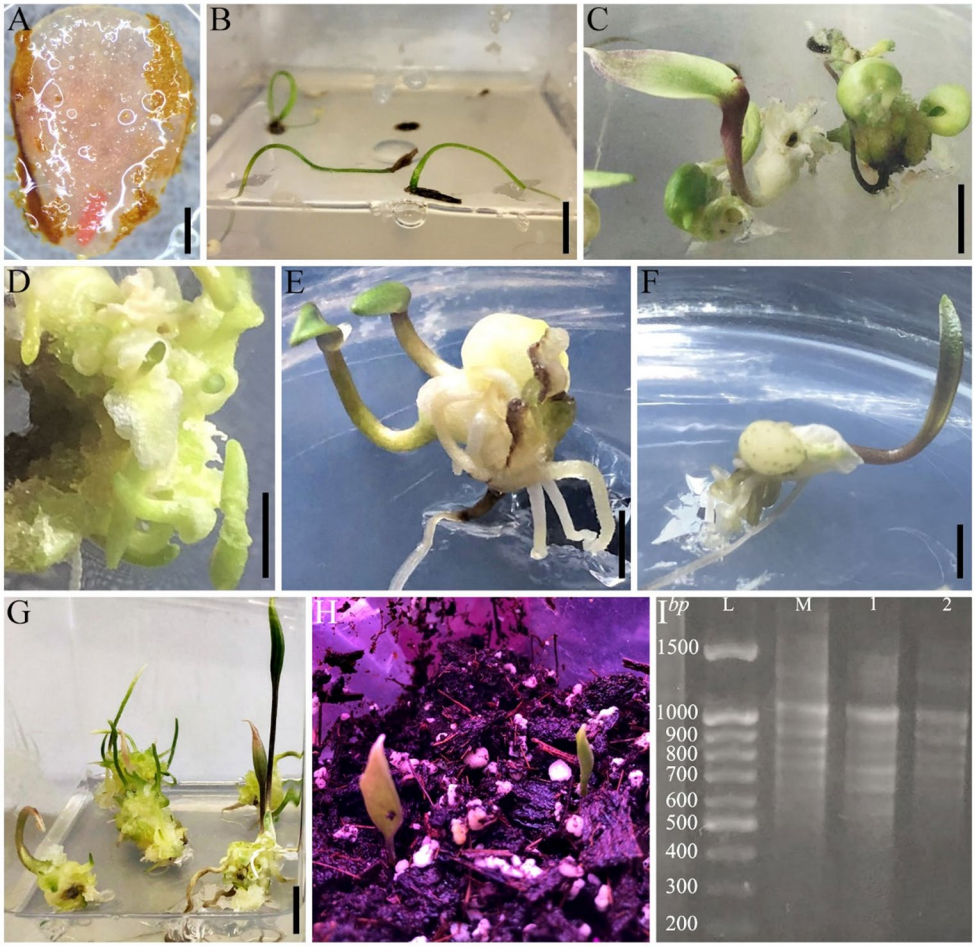



**Fig. 2** The images from the in vitro propagation of *Fritillaria pontica*. The panels represent viable seed stained during TTZ test (**A**), germinating seeds on the culture medium with BAP at 2.0 mg $L^{-1}$ after PCT (**B**), the shoots emerged from bulblets with minimal callus formation (**C**), caulogenesis from callus tissue cultured on MS medium (**D**), rooting response of the bulblets cultured on the medium with IBA at 1.0 mg $L^{-1}$ (**E** & **F**), the callogenesis response of bulblets on the medium with IBA at 0.5 mg $L^{-1}$ at the end of 6 month (**G**) the plants acclimatized to soil conditions after 3 weeks (**H**), and the ISSR profiles of the mother plant and the in vitro-propagated plants using UBC-866 primer (**I**). The labels in panel I indicate base pairs (bp), DNA ladder (L), plant from the control medium (M), and regenerants from the medium with 2.0 mg $L^{-1}$ (1) and 4.0 mg $L^{-1}$ KIN (2). The scale bars correspond to 0.1 cm in panel A and 1 cm in the other panels

weak developmental changes in rhizogenesis. IAA and IBA treatments at 0.5 mg $L^{-1}$ induced a rooting response (0.06 roots per seed) in the seeds treated with RSCT. In the ICT group, only IAA and NAA treatments at 1.0 and 2.0 mg $L^{-1}$, respectively, induced the same rooting response (0.06 roots per seed). Root lengths ranged from 0.01 to 0.04 cm per seed. In the RSCT group, IAA and IBA at 0.5 mg $L^{-1}$ induced roots with lengths of 0.01 and 0.02 cm per seed, respectively. None of the PGR treatments tested during SCT and PCT induced a rooting response.

Bulblet formation during SCT incubation occurred only in the presence of BAP and TDZ at a concentration of 2.0 mg $L^{-1}$ in the culture medium. In the RSCT group, in addition to the effective PGRs on bulblet formation during SCT, BAP at 0.25, KIN at 0.5, and IBA at 0.5-1.0 mg $L^{-1}$ induced bulblet formation. High concentration of BAP treatments and KIN treatments at 0.25-1.0 mg $L^{-1}$, IAA at 1.0, and IBA at 0.25 mg $L^{-1}$ induced bulblet formation during ICT treatment. The PCT treatment had the highest bulblet formation (0.26 bulblet per seed) in the medium containing 2.0 mg $L^{-1}$ BAP. TDZ treatment at 1.0 and 2.0 mg $L^{-1}$, BAP treatments at low concentrations, KIN at 2.0, and IAA at 0.25 mg $L^{-1}$ induced bulblet formation during PCT treatment (Table 5).

## Effects of PGRs on organogenesis from bulblets and calli

Two months after incubation, bulblets planted in the control medium showed moderate organ formation, while shoot and root development remained minimal. The TDZ-enriched medium at 1.0 mg $L^{-1}$ significantly increased organ formation from bulblets. In contrast, the medium with NAA and TDZ at 0.5 mg $L^{-1}$ yielded lower responses than that of the control and resulted in minimal organ and callus formation (Fig. 2C). At the end of 6 months of incubation, bulblets showed developed organ formation and increased shoot and root numbers. However, the most striking result was observed after 0.5 mg $L^{-1}$ TDZ treatment, which induced organogenesis in all bulblets and yielded the same shoot number as the control, despite no root development being recorded (Table 6). Callus formation was observed in all bulblet cultures, and increased growth was observed after 6 months of incubation. In particular, bulblets cultured in the medium with TDZ at 0.5 mg $L^{-1}$ formed 100% callus. Therefore, these calli were transferred to subcultures to promote organ formation.

**Table 2** The effects of plant growth regulators on in vitro seed germination in *Fritillaria pontica* under different temperature regimes and incubation periods. The data indicate the mean percentage of the seeds germinated (%) with standard error

| Treatment | Temperature & period | | | |
|---|---|---|---|---|
| | SCT | RSCT | ICT | PCT |
| Control | 0±0 b | 46.7±6.7 ab | 0±0 b | 0±0 b |
| TDZ 0.25 | 0±0 b | 33.3±13.3 bc | 13.3±6.7 ab | 0±0 b |
| TDZ 0.5 | 0±0 b | 46.7±6.7 ab | 13.3±6.7 ab | 0±0 b |
| TDZ 1.0 | 0±0 b | 20.0±0 cd | 6.7±6.7 ab | 20.0±0 a |
| TDZ 2.0 | 13.3±6.7 a | 60.0±0 a | 0±0 b | 20.0±0 a |
| BAP 0.25 | 6.7±6.7 ab | 33.3±6.7 bc | 0±0 b | 6.7±6.7 b |
| BAP 0.5 | 0±0 b | 13.3±6.7 cd | 6.7±6.7 ab | 6.7±6.7 b |
| BAP 1.0 | 0±0 b | 20.0±0 cd | 6.7±6.7 ab | 0±0 b |
| BAP 2.0 | 13.3±6.7 a | 13.3±6.7 cd | 20.0±0 a | 26.7±6.7 a |
| KIN 0.25 | 0±0 b | 20.0±0 cd | 13.3±6.7 ab | 0±0 b |
| KIN 0.5 | 0±0 b | 13.3±6.7 cd | 13.3±6.7 ab | 0±0 b |
| KIN 1.0 | 0±0 b | 0±0 d | 20.0±0 a | 0±0 b |
| KIN 2.0 | 0±0 b | 0±0 d | 0±0 b | 6.7±6.7 b |
| IAA 0.25 | 6.7±6.7 ab | 13.3±6.7 cd | 0±0 b | 6.7±6.7 b |
| IAA 0.5 | 0±0 b | 13.3±6.7 cd | 0±0 b | 0±0 b |
| IAA 1.0 | 0±0 b | 6.7±6.7 d | 13.3±6.7 ab | 0±0 b |
| IAA 2.0 | 6.7±6.7 ab | 46.7±6.7 ab | 6.7±6.7 ab | 6.7±6.7 b |
| IBA 0.25 | 6.7±6.7 ab | 13.3±6.7 cd | 13.3±6.7 ab | 0±0 b |
| IBA 0.5 | 0±0 b | 33.3±6.7 bc | 13.3±6.7 ab | 0±0 b |
| IBA 1.0 | 0±0 b | 53.3±6.7 ab | 0±0 b | 0±0 b |
| IBA 2.0 | 13.3±6.7 a | 53.3±6.7 ab | 6.7±6.7 ab | 0±0 b |
| NAA 0.25 | 0±0 b | 0±0 d | 0±0 b | 0±0 b |
| NAA 0.5 | 0±0 b | 0±0 d | 0±0 b | 0±0 b |
| NAA 1.0 | 0±0 b | 20.0±11.5 cd | 0±0 b | 6.7±6.7 b |
| NAA 2.0 | 6.7±6.7 ab | 6.7±6.7 d | 13.3±6.7 ab | 0±0 b |

Means with the same letters in the same column are not statistically different from each other according to Duncan's multiple range test ($p<0.05$). The treatment codes indicate the PGR abbreviation and the concentration tested in mg $L^{-1}$

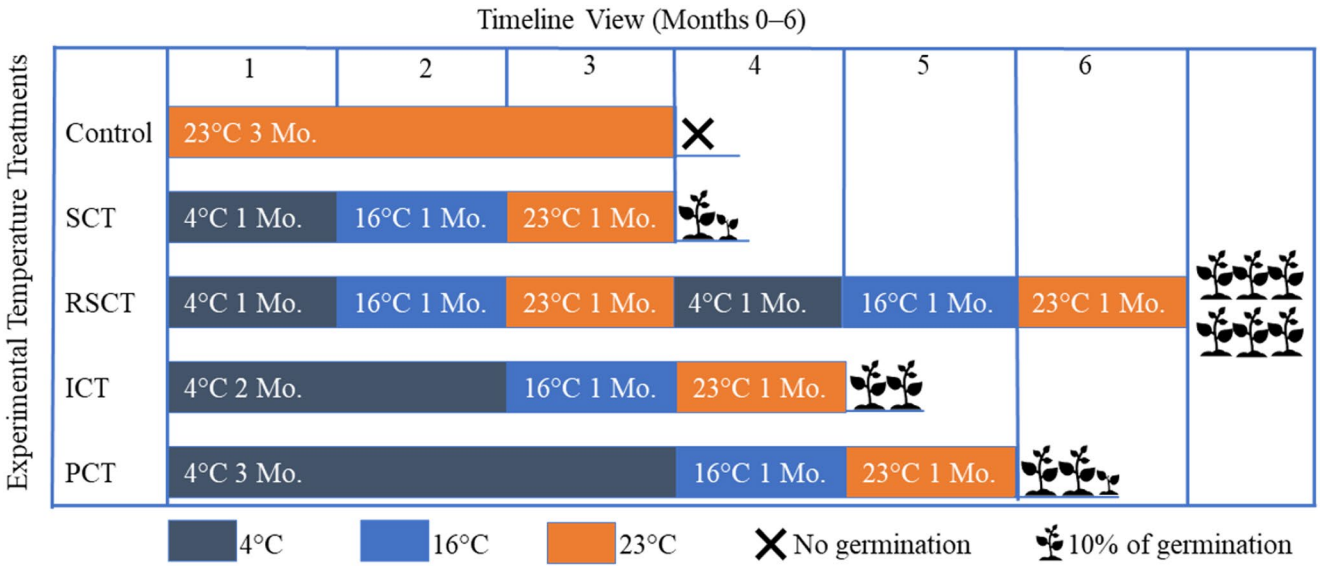



**Fig. 3** Graphical illustration of the incubation periods across different temperature regimes tested for in vitro seed germination in the presence of PGRs in *Fritillaria pontica*

**Table 3** The effects of plant growth regulators on in vitro shoot production in *Fritillaria pontica* seedlings under different temperature regimes and incubation periods. The data indicate the mean number of shoots per inoculated seed with standard error

| Treatment | Temperature & period | | | |
|---|---|---|---|---|
| | SCT | RSCT | ICT | PCT |
| Control | 0±0 b | 0.46±0.06 ab | 0±0 b | 0±0 b |
| TDZ 0.25 | 0±0 b | 0.26±0.06 cd | 0.13±0.06 b | 0±0 b |
| TDZ 0.5 | 0±0 b | 0.46±0.06 ab | 0.13±0.06 b | 0±0 b |
| TDZ 1.0 | 0±0 b | 0.2±0 cde | 0.06±0.06 b | 0.2±0 a |
| TDZ 2.0 | 0.13±0.06 a | 0.6±0 a | 0±0 b | 0.2±0 a |
| BAP 0.25 | 0.06±0.06 ab | 0.33±0.06 bc | 0±0 b | 0.06±0.06 b |
| BAP 0.5 | 0±0 b | 0.13±0.06 def | 0.06±0.06 b | 0.06±0.06 b |
| BAP 1.0 | 0±0 b | 0.2±0 cde | 0.06±0.06 b | 0±0 b |
| BAP 2.0 | 0.13±0.06 a | 0.13±0.06 def | 0.2±0 a | 0.26±0.06 a |
| KIN 0.25 | 0±0 b | 0.2±0 cde | 0.13±0.06 b | 0±0 b |
| KIN 0.5 | 0±0 b | 0.13±0.06 def | 0.13±0.06 b | 0±0 b |
| KIN 1.0 | 0±0 b | 0±0 f | 0.2±0 b | 0±0 b |
| KIN 2.0 | 0±0 b | 0±0 f | 0±0 b | 0.06±0.06 b |
| IAA 0.25 | 0.06±0.06 ab | 0.13±0.06 def | 0±0 b | 0.06±0.06 b |
| IAA 0.5 | 0±0 b | 0.13±0.06 def | 0±0 b | 0±0 b |
| IAA 1.0 | 0±0 b | 0.06±0.06 ef | 0.13±0.06 b | 0±0 b |
| IAA 2.0 | 0.06±0.06 ab | 0.46±0.06 ab | 0.06±0.06 b | 0.06±0.06 b |
| IBA 0.25 | 0.06±0.06 ab | 0.13±0.06 def | 0.13±0.06 b | 0±0 b |
| IBA 0.5 | 0±0 b | 0.33±0.06 bc | 0.13±0.06 b | 0±0 b |
| IBA 1.0 | 0±0 b | 0.53±0.06 a | 0±0 b | 0±0 b |
| IBA 2.0 | 0.13±0.06 a | 0.53±0.06 a | 0.06±0.06 b | 0±0 b |
| NAA 0.25 | 0±0 b | 0±0 f | 0±0 b | 0±0 b |
| NAA 0.5 | 0±0 b | 0±0 f | 0±0 b | 0±0 b |
| NAA 1.0 | 0±0 b | 0.2±0.11 cde | 0±0 b | 0.06±0.06 b |
| NAA 2.0 | 0.06±0.06 ab | 0.06±0.06 ef | 0.66±0.33 a | 0±0 b |

Means with the same letters in the same column are not statistically different from each other according to Duncan's multiple range test ($p<0.05$). The treatment codes indicate the PGR abbreviation and the concentration tested in mg $L^{-1}$

The calli cultured on the control medium had a higher organ formation rate and shoot and root production than those of the KIN treatments (Fig. 2D). The calli were white and yellow in color, mostly friable. After 6 months of incubation, the organ formation rate increased significantly. KIN treatment at 2.0 mg $L^{-1}$ had the highest organ formation rate (Table 7). In the same fashion as the shorter incubation period, shoot and root production significantly decreased in response to KIN treatments. The calli turned green and white in color but remained friable.

## Effects of IBA and incubation period on rhizogenesis

The shoots generated in callus cultures were then subjected to rooting experiments. On the 45th day of the rooting experiment, leaf-like structures began to develop in the apical region of some of the cultured shoots, and callus structures were observed in their lower parts. However, no organ formation was detected at the end of a 2-month incubation period. After a 6-month incubation period, root formation increased significantly (Fig. 2E-F). The highest root and shoot growth was observed in the medium with IBA 2.0 mg $L^{-1}$, whereas the lowest organ development was recorded from the medium with IBA 1.0 mg $L^{-1}$. The medium with IBA 0.5 mg $L^{-1}$ yielded the highest bulblet production (Table 8). During the long-term rooting period, IBA at 2.0 mg $L^{-1}$ induced the highest callus production, with fragile, green calli (Fig. 2G).

## Acclimatization success

After the filtered lids were mounted on the culture vessels, all the plants survived. The results indicated that 100% of the in vitro-raised plants with bulblets successfully survived 2 months in the soil to which they were transferred (Fig. 2H). However, the foliar parts of some of the transferred plants lost vitality after extended periods in the same soil under laboratory conditions, whereas the bulbs remained viable.

## Genetic fidelity of the regenerants

Two randomly selected plant samples regenerated from callus cultures were subjected to PCR to assess genetic fidelity in in vitro-developed plants. Among the primers,

**Table 4** The effects of plant growth regulators on in vitro shoot elongation in *Fritillaria pontica* seedlings under different temperature regimes and incubation periods. The data indicate the mean shoot length (cm) per inoculated seed with standard error

| Treatment | Temperature & period | | | |
|---|---|---|---|---|
| | SCT | RSCT | ICT | PCT |
| Control | 0 ± 0 c | 0.50 ± 0.23 abcd | 0 ± 0 b | 0 ± 0 c |
| TDZ 0.25 | 0 ± 0 c | 0.16 ± 0.11 bcd | 0.47 ± 0.23 b | 0 ± 0 c |
| TDZ 0.5 | 0 ± 0 c | 0.47 ± 0.08 abcd | 0.66 ± 0.37 b | 0 ± 0 c |
| TDZ 1.0 | 0 ± 0 c | 0.32 ± 0.04 abcd | 0.26 ± 0.26 b | 0.55 ± 0.14 bc |
| TDZ 2.0 | 0.28 ± 0.14 ab | 0.84 ± 0.23 a | 0 ± 0 b | 0.64 ± 0.07 b |
| BAP 0.25 | 0.14 ± 0.14 bc | 0.67 ± 0.17 ab | 0 ± 0 b | 0.34 ± 0.34 bc |
| BAP 0.5 | 0 ± 0 c | 0.1 ± 0.08 cd | 0.04 ± 0.04 b | 0.36 ± 0.36 bc |
| BAP 1.0 | 0 ± 0 c | 0.10 ± 0.04 cd | 0.23 ± 0.23 b | 0 ± 0 c |
| BAP 2.0 | 0.4 ± 0.23 a | 0.4 ± 0.23 abcd | 0.70 ± 0.05 b | 1.46 ± 0.53 a |
| KIN 0.25 | 0 ± 0 c | 0.08 ± 0.04 cd | 0.20 ± 0.15 b | 0 ± 0 c |
| KIN 0.5 | 0 ± 0 c | 0.27 ± 0.14 bcd | 0.34 ± 0.17 b | 0 ± 0 c |
| KIN 1.0 | 0 ± 0 c | 0 ± 0 d | 0.65 ± 0.08 b | 0 ± 0 c |
| KIN 2.0 | 0 ± 0 c | 0 ± 0 d | 0 ± 0 b | 0.28 ± 0.28 bc |
| IAA 0.25 | 0.1 ± 0.1 bc | 0.21 ± 0.10 bcd | 0 ± 0 b | 0.2 ± 0.2 bc |
| IAA 0.5 | 0 ± 0 c | 0.16 ± 0.12 bcd | 0 ± 0 b | 0 ± 0 c |
| IAA 1.0 | 0 ± 0 c | 0.01 ± 0.01 d | 0.4 ± 0.20 b | 0 ± 0 c |
| IAA 2.0 | 0.08 ± 0.08 bc | 0.38 ± 0.15 abcd | 0.20 ± 0.20 b | 0.01 ± 0.01 c |
| IBA 0.25 | 0.1 ± 0.1 bc | 0.18 ± 0.13 bcd | 0.29 ± 0.15 b | 0 ± 0 c |
| IBA 0.5 | 0 ± 0 c | 0.84 ± 0.25 a | 0.27 ± 0.13 b | 0 ± 0 c |
| IBA 1.0 | 0 ± 0 c | 0.85 ± 0.50 a | 0 ± 0 b | 0 ± 0 c |
| IBA 2.0 | 0.09 ± 0.07 bc | 0.6 ± 0.16 abc | 0.02 ± 0.02 b | 0 ± 0 c |
| NAA 0.25 | 0 ± 0 c | 0 ± 0 d | 0 ± 0 b | 0 ± 0 c |
| NAA 0.5 | 0 ± 0 c | 0 ± 0 d | 0 ± 0 b | 0 ± 0 c |
| NAA 1.0 | 0 ± 0 c | 0.07 ± 0.03 cd | 0 ± 0 b | 0.06 ± 0.06 c |
| NAA 2.0 | 0.01 ± 0.01c | 0.01 ± 0.01 d | 1.5 + 0.86 a | 0 ± 0 c |

Means with the same letters in the same column are not statistically different from each other according to Duncan's multiple range test ($p < 0.05$). The treatment codes indicate the PGR abbreviation and the concentration tested in mg $L^{-1}$

UBC-859, 866, and 899 generated clear bands. The list of ISSR primers that produced scorable bands, together with their sequences, primer annealing temperatures, and amplified fragment ranges, is given in Table 9. Twelve capable bands were produced by the assessed primers, with 2 to 6 bands per primer. The amplified fragments ranged in size from 300 to 1700 bp. The amplicons were monomorphic, as the band patterns were similar across all groups (Fig. 2I).

### Hierarchical cluster analysis

The analysis classified the treatments based on the differences and similarities in their effects on plant development across different incubation periods. The analysis revealed two main clusters, depending on the PGR effect and incubation period. The highest-performing treatment, 2.0 mg $L^{-1}$ BAP, stood out for high germination, bulb formation, and shoot development and was found to be standalone in the main cluster. NAA and TDZ treatments at 2.0 mg $L^{-1}$ were placed alone in separate subclusters due to high germination and root and shoot development during the ICT and RSCT periods, respectively. NAA treatments at 0.25 and 0.5 mg $L^{-1}$ were placed in the same subcluster, which was the lowest-performing group. The IBA treatments at 0.5 and 1.0 mg $L^{-1}$ were in the same subcluster, whereas the control group was alone in a separate subcluster (Fig. 4).

## Discussion

Habitat destruction, climate change, and the global biodiversity crisis caused by human activities have made the conservation of plant species a critical priority (Muluneh 2021). Fritillaria seeds, after dispersing from the capsules of this family, exhibit greater difficulty in in vitro germination due to their germination period and seed dormancy (Tao et al. 2024; Li et al. 2025). These seeds remain dormant throughout summer and fall in their natural habitats, essentially waiting for the seasonal cycle to pass. They then emerge from dormancy in winter and begin germinating toward the end of the cold season, thereby synchronizing their growth processes with the optimal environmental conditions (Sheikh et al. 2022; Marković et al. 2023; Sharma et al. 2023). Consequently, simulating natural environmental conditions is

**Table 5** The effects of plant growth regulators on in vitro bulblet production in *Fritillaria pontica* seedlings under different temperature regimes and incubation periods. The data indicate the mean number of bulblets per inoculated seed with standard error

| Treatment | Temperature & period | | | |
|---|---|---|---|---|
| | SCT | RSCT | ICT | PCT |
| Control | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| TDZ 0.25 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| TDZ 0.5 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| TDZ 1.0 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0.06 ± 0.06 b |
| TDZ 2.0 | 0.06 ± 0.06 b | 0.06 ± 0.06 ab | 0 ± 0 c | 0.20 ± 0 a |
| BAP 0.25 | 0 ± 0 c | 0.06 ± 0.06 ab | 0 ± 0 c | 0.06 ± 0.06 b |
| BAP 0.5 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0.06 ± 0.06 b |
| BAP 1.0 | 0 ± 0 c | 0 ± 0 b | 0.06 ± 0.06 bc | 0 ± 0 b |
| BAP 2.0 | 0.13 ± 0.06 a | 0.13 ± 0.06 a | 0.2 ± 0 a | 0.26 ± 0.06 a |
| KIN 0.25 | 0 ± 0 c | 0 ± 0 b | 0.13 ± 0.06 ab | 0 ± 0 b |
| KIN 0.5 | 0 ± 0 c | 0.06 ± 0.06 ab | 0.13 ± 0.06 ab | 0 ± 0 b |
| KIN 1.0 | 0 ± 0 c | 0 ± 0 b | 0.2 ± 0 a | 0 ± 0 b |
| KIN 2.0 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0.06 ± 0.06 b |
| IAA 0.25 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0.06 ± 0.06 b |
| IAA 0.5 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| IAA 1.0 | 0 ± 0 c | 0 ± 0 b | 0.06 ± 0.06 bc | 0 ± 0 b |
| IAA 2.0 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| IBA 0.25 | 0 ± 0 c | 0 ± 0 b | 0.06 ± 0.06 bc | 0 ± 0 b |
| IBA 0.5 | 0 ± 0 c | 0.06 ± 0.06 ab | 0 ± 0 c | 0 ± 0 b |
| IBA 1.0 | 0 ± 0 c | 0.13 ± 0.06 a | 0 ± 0 c | 0 ± 0 b |
| IBA 2.0 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| NAA 0.25 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| NAA 0.5 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| NAA 1.0 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |
| NAA 2.0 | 0 ± 0 c | 0 ± 0 b | 0 ± 0 c | 0 ± 0 b |

Means with the same letters in the same column are not statistically different from each other according to Duncan's multiple range test ($p < 0.05$). The treatment codes indicate the PGR abbreviation and the concentration tested in mg $L^{-1}$

**Table 6** The effects of plant growth regulators on in vitro organ production per bulblet of *Fritillaria pontica* under different incubation periods. The data indicate mean ± standard error

| Treatment | Organ development (%) | | Shoot number | | Root number | |
|---|---|---|---|---|---|---|
| | 2 months | 6 months | 2 months | 6 months | 2 months | 6 months |
| Control | 44.4 ± 11.1 ab | 66.7 ± 0 b | 0.78 ± 0.1 a | 2.11 ± 0.4 a | 0.22 ± 0.1 a | 0.77 ± 0.3 ab |
| TDZ 0.5 | 22.2 ± 11.1 b | 100 ± 0 a | 0.33 ± 0 ab | 2.11 ± 0.3 a | 0 ± 0 a | 0 ± 0 b |
| TDZ 1.0 | 66.7 ± 0 a | 66.7 ± 0 b | 0.78 ± 0.1 a | 1.33 ± 0.4 a | 0.11 ± 0.1 a | 0.11 ± 0.1 b |
| IBA 0.5 | 44.4 ± 11.1 ab | 66.7 ± 19.2 b | 0.44 ± 0.1 ab | 1.33 ± 0.7 a | 0 ± 0 a | 0.67 ± 0 ab |
| IBA 1.0 | 44.4 ± 11.1 ab | 55.5 ± 11.1 bc | 0.44 ± 0.1 ab | 1.44 ± 0.1 a | 0 ± 0 a | 0.66 ± 0.4 ab |
| NAA 0.5 | 22.2 ± 11.1 b | 77.8 ± 11.1 ab | 0.11 ± 0.1 b | 1.66 ± 0.2 a | 0.11 ± 0.1 a | 1.33 ± 0.2 a |
| NAA 1.0 | 33.3 ± 0 b | 33.3 ± 0 c | 0.77 ± 0.4 a | 0.77 ± 0.4 a | 0.11 ± 0.1 a | 0.66 ± 0.4 ab |

Means with the same letters in the same column are not statistically different from each other according to Duncan's multiple range test ($p < 0.05$). The treatment codes indicate the PGR abbreviation and the concentration tested in mg $L^{-1}$

**Table 7** The effects of kinetin treatments on in vitro organ development from *Fritillaria pontica* calli under different incubation periods. The data indicate mean ± standard error

| Treatment | Organ development (%) | | Shoot number | | Root number | | Callus structure and morphology | |
|---|---|---|---|---|---|---|---|---|
| | 2 months | 6 months | 2 months | 6 months | 2 months | 6 months | 2 months | 6 months |
| Control | 50 ± 5.8 a | 80 ± 0 b | 1.4 ± 0.08 a | 5.05 ± 0.5 a | 0.25 ± 0.05 a | 4.3 ± 0.9 a | CW-LY/F-C/•• | CW-LG/F/•••• |
| KIN 2.0 | 35 ± 5 ab | 95 ± 5 a | 0.4 ± 0 b | 2.05 ± 0.5 b | 0 ± 0 b | 0.8 ± 0.1 b | LY/F/•• | CW-LG/F/•••• |
| KIN 4.0 | 30 ± 5.8 b | 65 ± 5 c | 0.4 ± 0.08 b | 1.45 ± 0.3 b | 0.15 ± 0.09 ab | 0.65 ± 0.3 b | CW-LY/F/• | CW-LG/F/••• |

CW: Creamy-white, LG: Light green, LY: Light yellow, F: Friable, C: Compact. The symbols indicate the diameter of calli between 0–0.5 cm (•), 0.5–1.0 cm (••), 1.0–1.5 cm (•••), and above 1.5 cm (••••). The treatment codes indicate the PGR abbreviation and the concentration tested in mg $L^{-1}$

**Table 8** The effects of indole-3-butyric acid treatments on in vitro rooting, new leaf formation, and callus production in *Fritillaria pontica* shoots after 6 months of incubation. The data indicate mean ± standard error

| Treatment | Root number | Root Length (cm) | Leaf number | Leaf length (cm) | Bulb formation | Callus structure and morphology |
|---|---|---|---|---|---|---|
| Control | 1.93 ± 0.67 ab | 1.09 ± 0.22 ab | 3.2 ± 0.8 ab | 1.79 ± 0.26 a | 2.06 ± 0.29 a | CW-LG/F/••• |
| IBA 0.5 | 2.13 ± 0.13 ab | 0.84 ± 0.19 ab | 3.53 ± 0.58 ab | 1.69 ± 0.23 ab | 2.66 ± 0.37 a | CW-LG/F/•• |
| IBA 1.0 | 0.8 ± 0 b | 0.46 ± 0.06 b | 1.4 ± 0.2 b | 1.05 ± 0.08 b | 0.66 ± 0.06 b | CW-LG/F/•• |
| IBA 2.0 | 3.13 ± 0.63 a | 1.51 ± 0.46 a | 4.46 ± 1.36 a | 1.94 ± 0.19 a | 2.53 ± 0.37 a | LG/F/•••• |

CW: Creamy-white, LG: Light green, F: Friable, C: Compact. The symbols indicate the diameter of calli between 0–0.5 cm (•), 0.5–1.0 cm (••), 1.0–1.5 cm (•••), and above 1.5 cm (••••). The treatment codes indicate the PGR abbreviation and the concentration tested in mg $L^{-1}$

**Table 9** List of ISSR primers, their sequences, annealing temperatures, number of produced bands, and ranges of amplified fragments

| Primer code | Primer sequence (5'-3')[a] | Annealing temperature (°C) | Number of bands produced | Ranges of the amplified fragments (bp) |
|---|---|---|---|---|
| UBC-859 | TGTGTGTGTGTGTGTGRC | 50 | 4 | 300–800 |
| UBC-866 | CTCCTCCTCCTCCTCCTC | 50 | 6 | 500–1500 |
| UBC-899 | CATGGTGTTGGTCATTGTTCCA | 53 | 2 | 1000–1700 |

[a] Single letter abbreviations for a mixed-base position: Y=(C, T), R =(A, G)

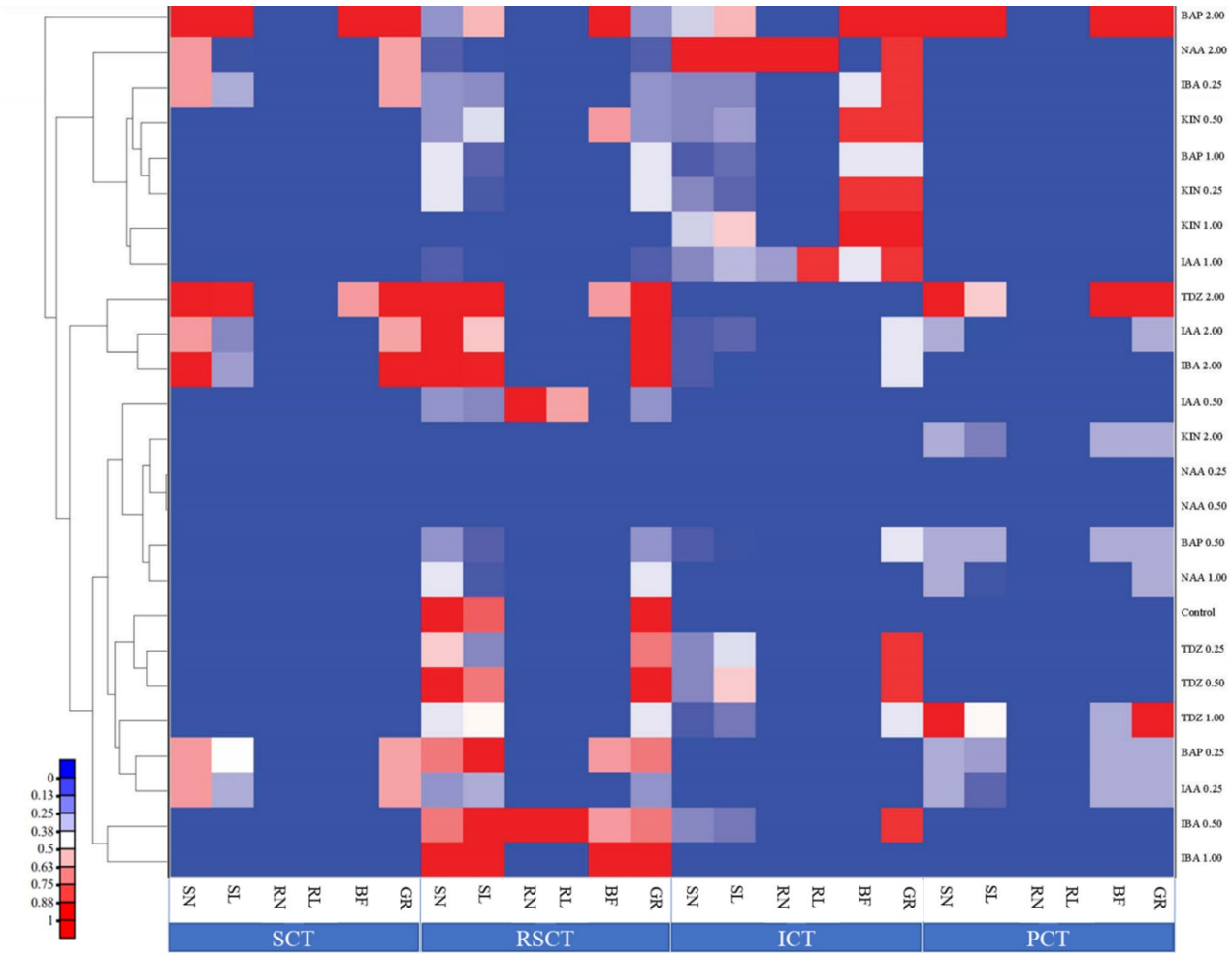



**Fig. 4** Heat map-assisted hierarchical classification of the plant growth regulator treatments and temperature regimes applied during different incubation periods based on their effects on developmental parameters measured in the propagation of *Fritillaria pontica*. SN: Shoot number, SL: Shoot length, RN: Root number, RL: Root length, BF: Bulblet formation, GR: Germination ratio

beneficial for accurately initiating the dormancy-release mechanism of seeds under in vitro conditions. Seeds of some species exhibit a deep state of dormancy, which also restricts their natural distribution and increases the risk of extinction.

The seed-oriented propagation method for Fritillaria species is complicated due to low germination rates and specific ecological or environmental conditions, and they are usually propagated via bulbs. However, due to their conservation status, only seeds were collected in our study, and efforts were made to increase their in vitro germination and organ development by testing the effects of PGRs and different incubation periods with cold treatments. Many geophytes and Fritillaria species require prolonged cold incubation to mimic their natural environmental conditions, end dormancy, and initiate germination (Marković et al. 2021). It has been reported that germination progressed more rapidly when cultures were exposed to alternating temperatures of 3 °C and 25 °C (Leubner-Metzger 2006). This reflects a climatic adaptation that ensures germination is synchronized with suitable seasonal conditions in natural habitats. High temperatures cause dormancy in geophytic species, while low temperatures break dormancy and promote growth and development (Petrić et al. 2013).

In our study, the higher germination rate in *F. pontica* recorded after the RSCT emphasized that such geophytes require a gradually increasing temperature regime and a long period of low temperatures throughout the year to complete their life cycle (Khodorova and Boitel-Conti 2013; Marković et al. 2021). Among the PGRs tested, TDZ and IBA treatments at specific concentrations were the most effective for germination in *F. pontica*. Our study demonstrated that cold incubation, combined with the addition of appropriate PGRs to the culture medium, significantly increased seed germination and helped break dormancy in *F. pontica* seeds. Similarly, in the study by Kizil and Khawar (2014), who cultured *F. persica* seeds in the dark at 4 °C on MS medium containing 2.0 mg $L^{-1}$ BAP and 1.0 mg $L^{-1}$ IBA, recorded a maximum seed dormancy rate (80%). However, they reposted that seeds exposed to longer cold incubation periods (3 months in the dark at 4 °C) exhibited limited germination, whereas the control group consistently failed to germinate under all conditions. Consequently, these findings underscore the importance of strategically using a long cold period followed by a gradual temperature increase during culture on a medium supplemented with appropriate PGRs to promote germination in Fritillaria species. Bhattacharya (2022) highlighted that seeds exposed to prolonged cold may exhibit poor germination and remain in secondary dormancy. In contrast, Kizil and Khawar (2014) reported that a 60-day incubation period at 10 °C, following at least 30 days at 4 °C, is necessary to break seed dormancy. Akyüz et al. (2018) incubated *F. imperialis* and *F. persica* seeds on MS medium at 4 °C for 3 months, then transferred them to 16 °C. They recorded germination rates of 73% for *F. imperialis* and 82.1% for *F. persica* seeds. Another study on *F. persica* reported a germination rate of 96.7% for immature seeds subjected to cold incubation for 3 months (Çakmak et al. 2016). These reports and our findings highlight the importance of further optimizing incubation periods and the duration of cold treatment. In another study, to break the dormancy of *F. reddula* seeds, the seeds were placed on wet filter paper in a Petri dish and incubated at 9 °C for 4 to 6 weeks, with germination observed approximately 4 months later. Additionally, in the same study, MS medium supplemented with 1% activated charcoal achieved the highest (60%) germination rate (Sadr et al. 2019). Furthermore, the time from germination to maturity of Fritillaria species varies with the composition of the culture medium, and their propagation via seed germination is very slow, as it may take up to 6 years to reach maturity (Sharma et al. 2023). These observations are consistent with this study's findings and emphasize the importance of optimal incubation periods for germinating Fritillaria species.

Our results showed that PGR concentrations and cold incubation duration significantly affected organ development outcomes. Very few seeds of Fritillaria species naturally produce bulbs, and it takes years for them to reach sufficient size, further limiting their cultivation using traditional methods. Therefore, the in vitro propagation studies offer promising alternatives to these methods. For example, Zhang et al. (2024) and Wang et al. (2025) demonstrated that tissue culture can significantly increase bulb yield, quality, and bioactive metabolite production in the endangered medicinal plant *F. cirrhosa*. These methods contribute to both the conservation of Fritillaria species and the development of sustainable cultivation methods that could support future commercial production.

In the current study, bulb production increased with the cold incubation period. Similarly, Akyüz et al. (2018) reported that bulb formation rates in *F. imperialis* and *F. persica* cultures were 76% and 82%, respectively, after cold incubation at 4 °C for 3 months, followed by transfer to 16 °C. Also, similar findings to the higher stimulatory effect of BAP treatment at a specific concentration on bulb formation in *F. pontica* under extended cold treatments were previously reported for *F. persica* and *F. ruthenica*, respectively, for bulb formation, bud induction, and shoot multiplication. Kizil and Khawar (2014) reported that more bulbs were obtained in MS medium containing 1.0 mg $L^{-1}$ BAP and IBA in *F. persica*, while Muraseva and Novikova (2018) found that the use of B5 medium (Gamborg et al. 1968) supplemented with 5.0 µM BAP was the most effective method for adventitious bud induction and shoot multiplication in *F.*

*ruthenica*. This indicates that both the types and concentrations of the medium and PGRs, as well as the duration of cold treatment, significantly affect breaking dormancy and bulb development in these species.

As shown in the cluster map in our study, cold treatments lasting 1 and 3 months at 4 °C reduced growth in all groups, highlighting the decisive effects of both PGR type and cold incubation duration on growth success. These results demonstrate that PGR applications are most effective under appropriate cold conditions.

In our study, we successfully established a methodology for de novo organogenesis in callus and bulb cultures of *F. pontica*. TDZ treatment promoted leaf and callus formation in *F. pontica*. Similarly, in a study by Petrić et al. (2012), the addition of TDZ to the culture medium of *F. meleagris* resulted in the formation of compact embryogenic callus, which demonstrated the efficacy of cytokinins such as TDZ in enhancing organ and callus formation in Fritillaria species. Furthermore, the effects of different auxin-cytokinin combinations on the in vitro morphogenesis of *F. meleagris* were investigated using bulb sections by Marković et al. (2023). On the other hand, Zhao et al. (2018) successfully developed an in vitro regeneration system by culturing *F. cirrhosa* bulb scales in MS medium containing 2.0 mg $L^{-1}$ BAP and 0.5 mg $L^{-1}$ NAA, and they demonstrated that the species can be propagated at a higher rate using vegetative materials.

Fritillaria species, bulb regeneration, callus induction, and direct organogenesis from plant tissues were demonstrated in previous studies through multiple pathways. For example, in *F. hupehensis*, efficient bulb formation has been demonstrated using embryonic callus structures (Shiau et al. 2000; Chen et al. 2000). Hao et al. (2017) enriched the MS medium with 2.0 mg $L^{-1}$ NAA and 0.5 mg $L^{-1}$ BAP for callus induction in *F. thunbergii*, and they reported that this auxin-dominant combination had a potent stimulatory effect on the species. In a study on *F. cirrhosa*, Chang et al. (2020) observed callus and bulb formation in the basal regions of germinated seedlings after adding 1.0 mg $L^{-1}$ BAP and 0.4 mg $L^{-1}$ NAA to the MS medium. In another study, Kumar et al. (2020) achieved highly efficient callus induction by adding 2.0 mg $L^{-1}$ picloram and 0.5 mg $L^{-1}$ TDZ to MS medium in *F. roylei*. Thus, optimizing explant type and culture medium, PGR type and concentration, and incubation time in in vitro propagation is crucial for both commercial and conservation purposes, as well as for sustainable production studies (Hasnain et al. 2022). Pasternak and Steinmacher (2024) reviewed two possible regeneration approaches in plants: new shoot induction via exogenous cytokinin treatment and culturing on a PGR-free medium. In hypocotyls cultured on a PGR-free medium, auxin sourced from the hypocotyl induces adventitious root formation, which subsequently can produce cytokinin. Then it stimulates auxin synthesis in the hypocotyl's apical region. Therefore, a similar physiological mechanism would explain the induced shoot and root formation in the control medium from bulblets and calli in our study, suggesting that cytokinin treatment of such explants in the species is unnecessary. The same researchers indicated that the N: P: K ratio of MS medium is suboptimal for auxin cofactors, resulting in excessive vegetative growth, hyperhydricity, and poor shoot formation (Pasternak and Steinmacher 2025). Therefore, evaluating alternative culture medium compositions in *F. pontica* cultures may enhance the efficiency and rate of plant development.

The rhizogenic response of seedlings varied significantly with the incubation period and the concentration of the auxin tested. In our experiments, although leaf-like structures began to develop in the apical region of some cultured bulblets, and callus structures were observed at the basal parts, no root formation was detected after 2 months of culture. Only seedlings cultured for 6 months were successfully rooted. In the study by Akyüz et al. (2018), bulblets obtained in vitro from *F. imperialis* and *F. persica* seeds were rooted in ½MS medium containing 0.5 mg $L^{-1}$ IBA, achieving rooting rates of 40% and 35.8%, respectively. Also, Çakmak et al. (2016) demonstrated that root formation occurs in *F. persica* seedlings using PGR combinations comprising BAP, NAA, kinetin, and TDZ. These findings emphasize that testing extended incubation periods and rooting media with different strengths, enriched with various PGR combinations, is beneficial for promoting root formation in *F. pontica* seedlings.

Based on the results of the primers tested, sufficient genetic stability was achieved in in vitro-regenerated plantlets derived from callus cultures in our study. Similarly, Muraseva et al. (2018) reported that *F. dagana* plants derived from in vitro cultures were genetically stable as assessed with ISSR markers.

Significant obstacles arose during the seedling's adaptation to *ex vitro* conditions. Foliar parts of some of these seedlings lost moisture and vitality after 2 months under laboratory conditions. This was most likely due to the leaves not being sufficiently mature for transfer to *ex vitro* conditions, or to bulbs without adequately developed root systems for proper water uptake. Paek and Murthy (2002) subjected *F. thunbergii* bulbs larger than 10 mm in diameter to cold treatment at 5 °C for 5 weeks, then planted them in a potting medium consisting of a mixture of peat moss, vermiculite, and perlite. Sprouting of the bulbs was observed within the subsequent 5 weeks. Accordingly, to ensure successful acclimatization and prevent loss of both aerial tissues and bulb organs, we recommend treating plants with cold and gradually increasing the temperature, allowing aboveground organs to emerge and, most importantly, maintaining

high bulb viability and productivity, thereby enhancing overall survival and establishment success. Also, seedlings can be treated with bacterial, fungal, and organic rooting stimulants to increase their transfer success to the external environment (Hyder et al. 2023). In addition, the addition of organic compounds and various salt formulations to the nutrient medium is important for optimizing bulb and root development in bulbous plants (Yasemin and Beruto 2024). These additions strengthen the plants, improve their adaptation to soil conditions, and enhance their ability to develop in new environments. Therefore, further research is needed to establish effective protocols for transferring Fritillaria species' bulbs to external environments.

## Conclusion

Various PGRs and their combinations were tested for in vitro germination of *F. pontica* seeds incubated under different temperature regimes. The results indicated that cold treatment successfully promotes seed germination. Especially, when seeds were cultured and then re-exposed to the same temperature cycle (RSCT; one month each at 4 °C, 16 °C, and 23 °C). In addition, the highest germination rate was generally obtained in media containing 2.0 mg $L^{-1}$ TDZ. This study also investigated the potential for organ formation in a KIN-containing medium using spontaneously formed callus structures from *F. pontica* bulb explants. The results emphasize that in vitro propagation and organ production are possible through callus cultures of *F. pontica*. Considering that this species contains antioxidant compounds of significant medicinal value, callus formation from in vitro-grown bulb explants can represent a highly effective approach for both conservation and secondary compound production. Finally, the regenerants should be further acclimatized to soil conditions by exposure to cold and gradually increasing temperatures, which would enhance the plants' survival in natural habitats. This step would increase the plant's presence in nature and support its long-term conservation.

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**Author contribution** All the authors contributed to the conception of the study. A.R. and A.A. designed the study. A.R. performed the experiments, data analysis, and statistical comparisons. A.R. and A.A. prepared the figures. F.Ö. obtained the official permission to collect the seeds. A.R. and A.A. wrote the manuscript. All authors commented on and contributed to the revision of the manuscript. All authors read and approved the final manuscript.

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**Data availability** All data generated or analyzed during this study are included in this published article and/or available from the corresponding author.

## Declarations

**Ethical approval** Not applicable.

**Competing interests** The authors have no relevant financial or non-financial interests to disclose.

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