Meta-Topolin mediated in vitro propagation in an ornamental important crop *Iris × hollandica* Tub. cv. professor Blaauw and genetic fidelity studies using SCoT markers

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**Abstract**

Dutch iris is a commercially important bulbous ornamental crop. Its high demand in global floriculture market necessitates the production of its high-quality planting material. In the present investigation, an efficient in vitro propagation system has been developed for *Iris × hollandica* Tub. cv. Professor Blaauw (Dutch iris) using meta-Topolin (mT) for the first time. Effect of various concentrations of BAP, Kn, and mT (0, 0.5, 1.0, 1.5 and 2 mg L⁻¹) along with varying photoperiods (16 h light and dark incubation for 1, 2, 3 and 4 weeks) on in vitro shoot induction from the twin scale explants was studied. Of the cytokinins tested, different doses of mT has resulted in better shoot induction response from twin scale explants than BAP and Kn. Photoperiod duration has also affected shoot induction response significantly. Along with dark incubation for 1 week, cytokinin mT at 1.0 mg L⁻¹ in MS medium has resulted in maximum shoot induction response (91.63%) with increased emergence of micro shoots (4.83 shoots/explant with average shoot length of 5.02 cm). Efficacy of BAP and mT alone or in combination with auxins for in vitro shoot multiplication was also compared. The synergistic effect of cytokinin-auxin in multiplication medium comprising MS + 1.0 mg L⁻¹ mT + 0.25 mg L⁻¹ NAA resulted in considerably higher number of shoots (17.53) with mean shoot length (7.06 cm) and maximum number of bulblets (2.74). Positive effect of increased sucrose concentration (90 g L⁻¹) alone or with paclobutrazol (5 mg L⁻¹) on in vitro bulblet formation and bulblet size was observed respectively. The superiority of mT over BAP was also found during in vitro rhizogenesis. Shoots raised on the mT medium were healthy and long enough, thus showed better rooting response (63.83%) on ½ MS medium + 0.5 mg L⁻¹ IAA after 4 weeks of incubation. About 89.16% survival rate was recorded for in vitro raised plantlets under ex vitro conditions. Analysis of clonal fidelity of thirteen in vitro regenerated plants was done using SCoT markers. Out of 36 primers, 13 primers showed clearly scorable monomorphic bands, thus displaying genetic uniformity among in vitro regenerated plantlets. This mT mediated protocol can be routinely used for the rapid large scale production of this valuable floriculture crop.

**Key message**

Reliable and consistent mT mediated protocol for in vitro regeneration of Dutch iris was established, which can be applied for rapid large scale production of this valuable floriculture crop and will also open up the way for genetic engineering/gene editing in iris and other bulbous crops.

**Keywords** Cytokinin · Floriculture crop · In vitro regeneration · Meta-Topolin · Photoperiod

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| mT/Meta-Topolin | 6-(3-Hydroxybenzylamino)purine |
| BAP | 6-Benzylaminopurine |
| Kn/Kinetin | 6-Furfurylaminopurine |
| NAA | α-Naphthyl acetic acid |
| PGRs | Plant growth regulators |
| MS | Murashige and Skoog |
| IAA | Indole 3-acetic acid |

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SCoT  Start Codon targeted
PGRs  Plant growth regulators

Introduction

Iris belongs to the Iridaceae family, known to contain several hundreds of attractive flowering plant species (Asgough et al. 2009). It is perennial evergreen or deciduous herb found globally in the temperate region (Britannica 2020). Most of the iris species are rhizomatous, bulbous, and rarely tuberous. Iris × hollandica commonly known as Dutch iris is an ornamentaly important monocotyledonous bulbous crop developed through interspecific hybridization between Iris tingitana from North America and Iris xiphium from Spain and Portugal (Keppel 1978). Due to its eye-catching incredible flower colors, Dutch iris is rated as one of the most appreciated flower species, and valuable cut flower in the floriculture industry (Uzun et al. 2014). The leaves, bulbs, and rhizomes of some species from Iridaceae family have been used in traditional and modern medicines, exhibiting properties such as anti-inflammatory, antiallergic, and respiratory tract disease (Rahman et al. 2003; Ayoub et al. 2018) and also used in cosmetic industry such as perfumes and soaps (Wang et al. 1999; Jevremovic and Radojevic 2002; Nasircilar and Deniz 2014).

The propagation of the iris through seedlings is difficult due to the poor rate of seed set and germination (Simonet 1932). Therefore, traditionally, irises are propagated by bulbs or rhizomes vegetatively which is very slow and inefficient, and can produce only three to five daughter bulbs per mother bulb per year (Hussey 1975; Jehan et al. 1994; Shibli and Ajlouni 2000). Moreover, the new bulbs produced vegetatively are most likely susceptible to the fungal, bacterial, and iris borer. Thus, to reduce the long and uncertain growth period of this bulbous crop and also to meet the high demand of quality planting material of this commercially important cultivar, alternative methods of propagation are required. Plant tissue culture is one of the promising technique for commercial propagation, as it offers a faster rate of multiplication and production of uniform planting material in a specified time period (Bhojwani and Dantu 2013). Micropropagation of several ornamental bulbous crops has been established earlier using twin-scale explants along with the basal plate tissue (Van der Linde et al. 1987; Fennell et al. 2004; Rice et al. 2011; Neube et al. 2015; Rahimi Khonakdari et al. 2020; Priyadharshini et al. 2020).

A major requirement for any explant to respond is type and concentration of plant growth regulators (PGRs) used in culture medium. Furthermore, among PGRs optimization of cytokinins governs the success of any in vitro regeneration protocol (Shekhwat et al. 2021). Use of cytokinins such as BAP (6-Benzylaminopurine) for in vitro organogenesis is well characterized and established in bulbous crops (Slabbert et al. 1993; Priyadharshini et al. 2020). However, a newly developed aromatic cytokinins such as mT {6-(3-Hydroxybenzylamino)purine} act as a prolific substitute against BAP and considered as a panacea against several bottlenecks such as shoot tip necrosis, hyperhydricity, senescence, reduced and deformed growth in plant tissue culture (Aremu et al. 2012). The beneficial effect of mT was also demonstrated for improved multiplication rate, shoot quality, induction of in vitro rooting and acclimatization (Lata et al. 2016; Jayaprakash et al. 2021).

In bulbous crops, inspite of in vitro shoot induction and shoot proliferation, in vitro bulblet formation is also necessary as the in vitro bulblets are easier to handle, store, and transport. In fact, bulblets with larger sizes are usually preferred, as they have better chances for leaf emergence and increased rate of survival after its ex vitro transfer from in vitro conditions (Podwyszyńska 2012). Therefore, for speeding up the in vitro breeding process, efforts to increase in vitro bulblet number and size are needed. The supplementation of bulblets with sucrose and growth retardants like paclobutrazol, cycocyl (CCC), and abscisic acid (ABA) exogenously in the culture medium affects the bulb size under in vitro conditions (Nower 2007; Ptak 2014).

Previous studies carried out on the development of micropropagation protocol in iris species (Van der Linde et al. 1987; Nower 2007; Hussey 1976; Yabuya et al. 1991; Zhu et al. 2015; Wang et al. 2018) concluded that this plant can’t be micro propagated on a larger scale due to the various hurdles during in vitro morphogenesis and in vitro rooting. So far, no study has included any information on the application of mT for in vitro organogenesis in Dutch iris. Therefore, this study aimed to develop mT mediated reliable in vitro propagation system in Dutch iris to generate quality planting material and to determine the genetic fidelity of regenerated plantlets using SCoT markers. The applicability of the developed protocol for this commercial variety can be used for other cultivated and wild varieties of iris with slight modifications. Moreover, this will open up the way for genetic engineering/gene editing in iris and other bulbous crops to create new varieties with characteristics such as novel flower colour, scented flower, prolonged flower vase-life, biotic and abiotic resistance (Teixeira da Silva et al. 2013; Noman et al. 2017).

Materials and methods

Plant material and disinfection of bulbs

Healthy bulbs of Iris × hollandica Tub. cv. Professor Blaauw were collected from the field of Floriculture,
Throughout the study, MS (Murashige and Skoog, 1962) basal medium comprising of macro, micronutrients, vitamins, 3% (w/v) sucrose as carbon source (HiMedia, India) and 0.8% (w/v) agar as solidifying agent (HiMedia, India) was used. PGRs such as 6-benzylaminopurine (BAP), 6-furfurylaminopurine (Kinetin/Kn), 6-(3-hydroxybenzylamino) purine (meta-Topolin/mT), α-naphthileneacetic acid (NAA) and Indole 3-acetic acid (IAA) used in this study were bought from HiMedia, India. Prior to autoclaving, PGRs were added to MS medium and the final pH of the medium was adjusted to 5.8 using 0.1 N NaOH (sodium hydroxide) and 0.1 N HCl (hydrochloric acid). Culture medium of 100 ml was then poured into glass jars (300 ml) for in vitro shoot induction, shoot multiplication/proliferation and bulblet formation experiment, while for in vitro rooting experiment, glass culture tubes (25 × 150 mm, 100 ml, Borosil, India) filled with 20 ml of medium were used. These culture vessels containing medium were sterilized in autoclave at 121 °C for 20 min. The culture room was maintained at 25 ± 2 °C, 16 hour (h) photoperiod using cool-white fluorescent tube lights (Phillips, Netherlands) with a photon flux density of 35 μmol m⁻² s⁻¹, and relative humidity was maintained up to 70%. For dark incubation, culture flasks covered with black paper were placed in clean cardboard box.

### Media and culture conditions

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### Explants preparation and in vitro shoot induction

With the help of surgical blade, the surface sterilized bulbs were dissected longitudinally to get 6 twin-scales explants (3 cm long). To study the effect of cytokinins on the in vitro shoot induction, explants preparation and in vitro shoot induction table was created. The table includes the shoot induction (%), number of shoots, and shoot length (cm) for different cytokinins (BAP, Kn and mT) and photoperiod (16 h L, 1 wk D, 2 wk D, 3 wk D, 4 wk D). The results show that BAP 2.0, Kn 2.0, and mT 2.0 concentrations were the most effective in promoting shoot induction and shoot multiplication/proliferation. The shoot length was also measured and the highest shoot length was observed with BAP 2.0, Kn 2.0, and mT 2.0 concentrations. The photoperiod of 16 h L, 1 wk D, 2 wk D, 3 wk D, and 4 wk D also played a significant role in shoot induction and shoot multiplication/proliferation. The results indicate that a photoperiod of 16 h L, 1 wk D, 2 wk D, 3 wk D, and 4 wk D promoted the highest shoot induction and shoot multiplication/proliferation.

### Table: Shoot induction (%), number of shoots, and shoot length (cm)

| Cytokinins | 16h L 1 wk D 2 wk D 3 wk D 4 wk D | 16h L 1 wk D 2 wk D 3 wk D 4 wk D | 16h L 1 wk D 2 wk D 3 wk D 4 wk D |
|------------|----------------------------------|----------------------------------|----------------------------------|
| BAP 0.5    | 69.45 71.78 43.92 40.18 17.18    | 1.5 1.83 1.07 0.73 0.47          | 2.9 3.23 2.56 2.04 0.94         |
| BAP 1.0    | 47.22 49.22 35.89 32.63 11.67    | 1.33 1.52 1.21 1.12 0.83         | 2.38 2.89 2.34 1.89 0.85         |
| BAP 1.5    | 66.67 67.33 36.67 28.3 4.83      | 1.73 1.93 1.52 1.17 0.73         | 2.89 3.17 2.89 2.13 0.98         |
| BAP 2.0    | 55.55 58.19 40.15 17.67 8.37     | 1.48 1.53 1.2 1.07 1.18          | 2.04 2.43 2.01 1.6 0.86          |
| Kn 0.5     | 27.78 30.13 26.67 15.07 0         | 1.2 1.35 1.03 0.7 0              | 1.27 1.49 1.31 0.74 0            |
| Kn 1.0     | 22.22 28.39 27.78 11.93 0         | 1.07 1.22 1 0.67 0               | 1.17 1.18 1.08 0.71 0            |
| Kn 1.5     | 25 23.89 13.89 6.93 0             | 1.23 1.31 1 0.33 0               | 1.37 1.61 1.29 0.88 0            |
| Kn 2.0     | 16.67 38.74 36.11 18.23 8.37      | 1.05 1.15 0.67 0.4 0             | 0.94 1.11 1.01 0.94 0            |
| Mean       | 49.79 54.20 42.24 28.25 12.28      | 1.96 2.23 1.48 1.13 0.75          | 2.66 2.89 2.52 1.52 0.82          |
shoot organogenesis, each twin scale explant joined by 5 mm of the basal plate was cultured on MS supplemented with different concentrations (0.0, 0.5, 1.0, 1.5 and 2.0 mg L\(^{-1}\)) of BAP, Kn and mT respectively (Fig. 1). In the presence of cytokinins, in vitro shoot induction response of twin scale explants was also studied under varying photoperiod conditions including 16 h L, 1 wk D, 2 wk D, 3 wk D and 4 wk D. The detailed description of different photoperiods used in the study is given below. The twin scale explants were placed with adaxial side down on the tissue culture medium to make sure that the meristematic region of explant touch the medium. The culture media without any plant growth regulators was treated as control in this study. The data on percent shoot induction, number of shoots and shoot length (cm) was recorded, after 4 weeks of culture.

**Photoperiod**

(i) 16 h L = 16 h photoperiod for 4 weeks  
(ii) 1 wk D = 1 week dark followed by 16 h photoperiod for 3 weeks  
(iii) 2 wk D = 2 weeks dark followed by 16 h photoperiod for 2 weeks  
(iv) 3 wk D = 3 weeks dark followed by 16 h photoperiod for 1 week  
(v) 4 wk D = Complete dark for 4 weeks

**In vitro shoot proliferation and bulblet formation**

To confirm the synergistic interactive effect of auxins and cytokinins on in vitro shoot proliferation and bulblet formation, shoots (3–5 cm) developed from meristematic region of basal plate were isolated and transferred on to multiplication medium comprising MS medium augmented with optimal concentrations of cytokinins (BAP 0.5 mg L\(^{-1}\) and mT 1.0 mg L\(^{-1}\)) and varying concentrations of auxins viz., IAA and NAA (0.25 mg L\(^{-1}\) and 0.5 mg L\(^{-1}\)). These shoots were further allowed to multiply and elongate in vitro. The number of shoots, shoot length (cm) and number of bulblets formed were recorded after 4 weeks of culture.

**Effect of sucrose and paclobutrazol on in vitro bulblet formation and bulblet size**

At this step, in vitro shoots at the multiplication stage were excised and transferred to the fresh MS medium supplemented with different concentrations of sucrose (30, 60 and 90 g L\(^{-1}\)) alone or in combination with growth retardant paclobutrazol (5 mg L\(^{-1}\)) to study their effect on in vitro bulblet number and bulblet size. The number of bulblets formed per culture clump and bulblet diameter were measured after 4 weeks of culture.

**In vitro rooting and acclimatization of in vitro plantlets**

To compare the efficacy of BAP and mT on in vitro rhizogenesis, healthy and well elongated micro-shoots (4–5 cm) raised on the multiplication media augmented with optimal concentrations of cytokinins and auxins (BAP medium: MS medium + 0.5 mg L\(^{-1}\) BAP + 0.5 mg L\(^{-1}\) NAA and mT medium: MS medium + 1.0 mg L\(^{-1}\) mT + 0.25 mg L\(^{-1}\) NAA) were selected. BAP* and mT** medium derived shoots were excised and transferred onto half-strength MS medium alone and in combination with auxins (IAA) at low concentration of 0.5 mg L\(^{-1}\) and 3% (w/v) sucrose, solidified with 0.6% (w/v) Agar- Agar. The data on percent rooting, root number and root length (cm) were recorded after 4 weeks of culture.

After 4 weeks, rooted plantlets/bulblets were taken out from the culture tubes and transferred to plastic pots with autoclaved cocopeat and sand (1:1) mixture. All the plantlets were covered with perforated plastic bags and kept inside growth room with high humidity (80–90%) and 25 ± 2 °C temperature conditions. After two weeks, the polybags were gradually removed for the gradual acclimatization of the plantlets. The growth and survival of plants was observed at weekly intervals.

*BAP derived shoots- micro-shoots raised on multiplication medium comprising MS medium + 0.5 mg L\(^{-1}\) BAP + 0.5 mg L\(^{-1}\) NAA  
**mT derived shoots- micro-shoots raised on multiplication medium comprising MS medium + 1.0 mg L\(^{-1}\) mT + 0.25 mg L\(^{-1}\) NAA.

**Genetic fidelity analysis of in vitro regenerated plantlets of Dutch iris**

For genetic fidelity studies total genomic DNA was extracted from leaf tissues (100 mg) of the thirteen randomly selected in vitro regenerated plants by using Qiagen DNeasy plant mini kit (Qiagen India Pvt. Ltd.) as per manufacturer protocol. The quantity as well as purity of the isolated DNA samples was measured by using NanoDrop™ 2000/2000c, Thermo Fisher Scientific, USA. A total of 36 SCoT primers were screened out of which 13 primers were selected for genetic stability analysis. All the PCR reaction carried out with 50 ng (nano grams) template from total genomic DNA. Go Taq® Green Master mix 2X containing 400 µM (micro molar)dNTPs, 2 x DNA polymerase, and 3 mM (milli molar)MgCl\(_2\) along with 20 pmol (Pico mole) of primer, were used for 25 µl volume of PCR reaction. The PCR reaction program for SCoT primers were set up at 94 °C for 4 min. followed one cycle, 35 cycles at 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min. and, with a final extension of 72 °C for 5 min in Thermal Cycler (Applied Biosystems,
USA). After the PCR amplification 1 µl of 10× bromophenol blue dye was added to load the samples for loading into the gel. The amplified DNA bands were analysed on 1.8% agarose gel containing 1 µl of ethidium bromide and running 1× TAE buffer at 80v for 1–2 h. The size of the DNA fragments was estimated by using 100 bp ladder. Fragments of the DNA for each SCoT primer was visualized under UV light and photographed using gel documentation system (Molecular imager® Bio-Rad).

Experimental design and data analysis

All the experiments were conducted according to completely randomized design (CRD). Experiments of in vitro shoot induction, shoot proliferation and in vitro bulblet formation were carried out in three replications using five glass jars per replicate. Each glass jar with 3 explants was considered as a single experimental unit. Likewise, in vitro rooting experiment was also conducted in three replications per treatment with 15 explants per replicate. A single explant was cultured per culture test tube. The collected data were subjected to analysis of variance (ANOVA) using statistical package of SPSS (Version 17.0, IBM). The significance of treatment effects was determined by analysis of variance (ANOVA) and means were compared on the basis of critical difference (CD) calculated at 0.05% level of significance.

Results

Effect of different cytokinins and photoperiod on in vitro shoot induction

The MS media without any PGRs could not induce any response from the explants, and lead to necrosis, whereas media supplemented with different concentrations of the cytokinins were able to promote shoot induction from twin scale explants of Dutch iris. It was observed that meristematic cells of the basal plate of scales of bulb get activated for organogenesis by cytokinins treatment. The morphogenic response of explants (Fig. 1) evaluated at different concentrations of BAP, Knap and mT (0.5, 1.0, 1.5, 2.0 mg L⁻¹) indicated a noticeable effect of concentrations and type of cytokinin on shoot induction response. Of the various treatments, mT (1.0 mg L⁻¹) was found to be more effective than other cytokinins, thus explains its advantage over using BAP and Knap. At this level, the maximum number of shoots (4.40) per explant, with an average shoot length (5.65 cm) in 86.11% of the cultures were recorded, after 4 weeks of incubation at 16 h photoperiod (Fig. 1 and 2D–F). The results of the variance analysis revealed that cytokinins, photoperiod and their interaction had significant effects on the per cent shoot induction, number of shoots and shoot length (Table 1; Fig. 1). The results showed that there was a significant difference (P = 0.05) between treatments of photoperiod (16 h L, 1 wk D, 2 wk D, 3 wk D and 4 wk D) on the shoot induction (%), number of shoots and shoot length, so that the maximum percent shoot induction (54.20) and number of shoots (2.23) and shoot length (2.89 cm) were obtained with initial 1 wk D incubation (Fig. 1).

In the study of the interaction effect of cytokinins and photoperiod conditions, initial 1 wk dark (D) treatment, significantly enhanced percent shoot induction (91.63) with 5.10 average number of shoots per explant with average shoot length of 5.80 cm from the twin scale explants when cultured on mT (1.0 mg L⁻¹) rich shoot induction medium (Fig. 1). Moreover, initial short dark incubation of 1 wk reduced the incidence of necrosis by overcoming phenolic exudation from the explant. Longer dark incubation periods or continuous dark (4 wk D) resulted in reduced shoot induction response (33.11%) with 2.37 shoot number on the MS medium + 1 mg L⁻¹ mT. No shoot induction response was noticed on cytokinin-free medium or control in all the photoperiods.

Synergistic effect of cytokinin-auxin on in vitro shoot proliferation and in vitro bulblet formation

To further validate the superiority of mT over BAP on in vitro proliferation, micro-shoots obtained from twin scale explants in the shoot induction experiment was subcultured on MS medium supplemented with optimal concentrations of two selected cytokinins (BAP at 0.5 mg L⁻¹ and mT at 1.0 mg L⁻¹) alone or in combination with auxins (IAA and NAA) at 0.25 and 0.5 mg L⁻¹ concentration. Among both the cytokinins (BAP and mT), mT responded better for in vitro shoot proliferation. However, synergistic interactive effect of cytokinins (BAP and mT) with auxins (NAA and IAA) on in vitro shoot proliferation was also observed (Table 2). Moreover, overall mean values observed indicated that the explants respond better when the medium was enriched with different concentrations of NAA. It was observed that the shoots obtained on the culture medium augmented with combination of cytokinins (BAP and mT) and NAA are healthy, green and vigorous. However, shoots obtained from the medium with BAP and mT alone or in combination with IAA were weak and short in length. It was found that the number of shoots regenerated on the mT alone or mT in combination with auxins (NAA and IAA) were significantly higher than that of the number of shoots observed on the BAP alone or BAP with auxins. Results
presented in Table 2 and Fig. 2 (J-L) showed that among all the treatments, MS medium supplemented with 1.0 mg L⁻¹ mT + 0.25 mg L⁻¹ NAA was found to be most effective for in vitro shoot multiplication and bulblet formation with maximum number of shoots (17.53), highest shoot length (7.06 cm) and highest number of bulblets (2.74).

**Effect of sucrose and paclobutrazol on in vitro bulblet formation**

This experiment was conducted to study the effect of sucrose at different concentrations (30, 60, and 90 g L⁻¹) alone and in combination with 5 mg L⁻¹ paclobutrazol (a growth
inhibitor) on in vitro bulblet formation in Dutch iris. Data presented in Fig. 2N and 3A showed that higher number of bulblet formation was observed with increasing concentration of sucrose alone at 60 g L\(^{-1}\) and 90 g L\(^{-1}\) (3.49 and 3.91, respectively) as compared to MS medium with 30 g L\(^{-1}\) sucrose (2.52). Diameter of in vitro bulblets also showed significant increase at higher doses of sucrose. Upon using growth inhibitor (paclobutrazol at 5 mg L\(^{-1}\)) in MS medium along with various doses of sucrose (30, 60, and 90 g L\(^{-1}\)), slight reduction in bulblet number (2.06, 3.36, 3.44) was observed (Fig. 3A). But the presence of paclobutrazol has increased bulblet diameter significantly at higher sucrose dose in MS medium (60 and 90 g L\(^{-1}\)). The maximum bulblet diameter (2.28 cm) was observed in MS medium supplemented with 90 g L\(^{-1}\) + 5 mg L\(^{-1}\) paclobutrazol (Fig. 2N and 3B).

In vitro rhizogenesis and acclimatization of in vitro raised plantlets

In this experiment in vitro rooting response of the BAP and mT derived micro-shoots of Dutch iris was compared on \(\frac{1}{2}\) MS medium with (0.5 mg L\(^{-1}\) IAA) or without auxins. Addition of auxin in \(\frac{1}{2}\) MS medium has augmented in vitro rooting response from both BAP and mT derived shoots. mT derived micro-shoots, showed significant increase in rooting response on both \(\frac{1}{2}\) MS and \(\frac{1}{2}\) MS medium supplemented with 0.5 mg L\(^{-1}\) IAA as compared to BAP derived shoots. The highest percent rooting (63.83) and highest number of roots (3.42) with root length (4.48 cm) was recorded from mT derived shoots on \(\frac{1}{2}\) MS medium supplemented with 0.5 mg L\(^{-1}\) IAA after 4 weeks of transfer (Table 3; Fig. 2O). Whereas, in the case of BAP-derived micro-shoots, 35.83 per cent in vitro rooting with maximum number of roots (2.15) per micro-shoot having length (2.46 cm), were recorded in similar conditions and equimolar concentration of IAA. Thus mT-derived micro-shoots were found to be more efficient for in vitro rooting as compared to BAP.

After 4 weeks of transplantation of healthy well rooted plantlets/bulblets to plastic cups containing hardening mixture. Phenotypic alteration in plants was noted (Fig. 2Q). Formation of new leaves and increase in height of plants was observed at weekly intervals. The maximum per cent survival rate of plants of Dutch iris in the greenhouse after 4 weeks was 89.16 with 15.24 cm long plants (Fig. 2P, Q, S1). The hardened plantlets were shifted to pots containing normal garden soil.

Assessment of genetic fidelity

Genetic fidelity analysis was carried out among the thirteen randomly selected in vitro regenerated plantlets, using SCoT
markers. A total of 36 SCoT primers were initially screened and finally 13 primers, produced clear, unambiguous and reproducible amplified bands (Table 4; Fig. 4). A total of 47 bands with an average of 4.06 bands per SCoT primer, among regenerants were scored and which were found to be monomorphic in banding pattern (Table 4). No sign of polymorphism was obtained during the SCoT analysis. The SCoT marker banding profiles produced by SCoT 15, SCoT 21 and SCoT 35 are shown in Fig. 4 A, B C respectively.

**Discussion**

In vitro regeneration systems are significantly affected by various factors such as genotype of plant, explant type, media composition (particularly PGRs), photoperiod and other culture conditions such as temperature etc. (Baranidiaran et al. 1999; Gantait et al. 2010; García-Fortea et al. 2020). Among all these factors, concentration of PGRs, and photoperiod are undoubtedly the most important factors.
that influence in vitro morphogenesis in the bulbous crops. Although previous studies have been documented for in vitro plantlet regeneration in various iris species through direct and indirect organogenesis (Van der Linde et al. 1987; Hussey 1976; Yabuya et al. 1991; Nower 2007; Meng et al. 2009; Ascough et al. 2009; Zhu et al. 2015; Wang et al. 2018). But to our knowledge, there is as yet no reliable regeneration system that discusses the application of cytokinin, mT in iris tissue culture. Thus, we have reported mT mediated in vitro propagation protocol using twin scale explants of Dutch iris.

Cytokinins plays crucial role for de novo shoot organogenesis in any plant species, as the appropriate use of optimal and effective cytokinins in tissue culture medium determines the morphogenetic competence (shoot/root organogenesis) of an explant. Our results showed that shoot induction response vary with the presence of different cytokinins in the regeneration medium. In the present study, mT was found more effective than BAP and Kn for multiple shoot bud induction in Dutch iris. Similar effect of mT on in vitro shoot/bulblet formation from the basal plate of the twin scale explants was reported in a bulbous crop named Malabar river lily (Crinum malabaricum) (Manokari et al. 2022). Several studies in various plant species have proved superiority of mT for in vitro shoot organogenesis and proliferation (Wojtania 2010; Aremu et al. 2012; Gentile et al. 2014; Ahmad and Anis 2019). In contrast to our results, maximum number of shoots from scale explants was observed in Iris hollandica in response to a combination of BAP (0.5 µM) and NAA (5.4 µM) (Hussey 1976). Application of BAP for in vitro organogenesis in bulbous crops have been reported (Rahimi Khonakdari et al. 2020). In Malabar river lily, an aquatic bulbous crop, MS medium augmented with 2.0 mg L⁻¹ BAP produced maximum 12 shoots per twin scale (Priyadharshini et al. 2020). Despite of its advantages as a potent cytokinin, in tissue culture, BAP has several shortcomings such as callus formation at the base of explant, problematic in vitro rooting, deformed growth among cultures, and physiological abnormality (Gentile et al. 2014).

Despite of cytokinins, photoperiod duration, quality (color and wavelength) and intensity of light is another critical factor for the growth and developmental process of plants under in vitro conditions (Bach et al. 2015). The effect of photoperiod on the in vitro regeneration of bulbous crops such as Lilium (Lilium longiflorum), Eucomis (Eucomis zambesiaca) and Narcissus (Narcissus tazzeta L.) has been reported (Niimi and Onozawa 1979; Cheesman et al. 2010; Rahimi Khonakdari et al. 2020). In Paulownia, the regeneration rate is significantly affected by different photoperiods under in vitro culture conditions (Yang et al. 2013). In the present study, initial dark incubation for 1 week lead to enhancement of shoot induction response as compared to 16 h photoperiod conditions for 4 weeks. Incubation of scale explants under dark conditions for four weeks under 20°C temperature conditions resulted in shoot induction, however, presence of light inhibited regeneration up to 40% from outer scale and inner twin scale explants of Dutch iris (Van der Linde et al., 1988). In various geophytes, dark conditions are suitable for the induction of bulblets (Rice et al. 2011; Bach et al. 2015). Continuous darkness increased the bulblet regeneration rate in Hyacinth (Hyacinthus orientalis) and Lilium (Kim et al. 1981). Conversely, maximum number of bulblets/leaves were recorded from the cultures kept in 16 h photoperiod in Narcissus was reported (Rahimi Khonakdari et al. 2020). Photoperiod intensity and duration are known to be inductive for the biosynthesis and degradation of cytokinins and lead to the change in the endogenous levels of cytokinins with in the explants which ultimately regulate the shoot induction response (Tapingkae and Taji 1999). Therefore, this concludes that requirement of different photoperiod for in vitro morphogenetic response is species and genotype specific.

The synergistic effect of cytokinin-auxin on in vitro shoot multiplication has been studied by various researchers in different plant species (Faisal et al. 2018; Ahmad and Anis 2019; Khanam et al. 2019; Gupta et al. 2020). For in vitro shoot multiplication, elongation and bulblet induction, optimized concentrations of cytokinins (BAP and mT) were tested in combination with various concentrations of different auxins (IAA and NAA). Similar to shoot bud induction, mT was found superior as compared to BAP for in vitro shoot proliferation as well. Moreover, mT (1.0 mg L⁻¹) interacted synergistically with auxin (NAA at 0.5 mg L⁻¹) and enhanced shoot proliferation as well as bulblet formation. In accordance with our findings, combinations between BA at 1.0 or 2.0 mg L⁻¹ and NAA at 0.2 mg L⁻¹ were the best medium for enhancing the number of shoots, shoot length and number of leaves.

Table 4 Response of different SCoT primers on assessment of genetic fidelity among in vitro regenerated plants of Iris × hollandica Tub. cv. Professor Blaauw

| S No | Primer code | Primer sequence (5′-3′) | Number of amplified bands |
|------|-------------|-------------------------|--------------------------|
| 1    | SCoT 12     | AGACCATGGCGACCACGCA    | 4                        |
| 2    | SCoT 15     | AGACCATGGCGACCGCCGA    | 5                        |
| 3    | SCoT 19     | ACCATGGCTACCAACGGCA    | 2                        |
| 4    | SCoT 21     | AGACCATGGCGACCGCCAC    | 5                        |
| 5    | SCoT 22     | AACCATGGCTACCACCCAC    | 3                        |
| 6    | SCoT 24     | CACCATGGCTACCAACCAT    | 4                        |
| 7    | SCoT 25     | ACCATGGCTACCAACCGTG    | 4                        |
| 8    | SCoT 27     | ACCATGGCTACCAACCGCG    | 3                        |
| 10   | SCoT 30     | CCATGGCTACCAACCGCC     | 3                        |
| 11   | SCoT 31     | CCATGGCTACCAACCGGC     | 3                        |
| 12   | SCoT 32     | CCATGGCTACCAACCGAG     | 4                        |
| 13   | SCoT 35     | CACATGGCTACCAACGGCC    | 5                        |

Total No. of bands 47
Enhanced in vitro shoot multiplication was recorded in different species of Lilium on the use of cytokinin with lower dose of auxin in the culture medium (Han et al. 2004; Youssef et al. 2019). Studies in different plant species such as *Mitragyna parvifolia* and *Maytenus emarginata* have also reported interactive effect of mT and auxin on in vitro shoot proliferation (Patel et al. 2020; Shekhawat et al. 2021). In contrast to our results, in Malabar River Lily, addition of auxins (IAA and NAA) in the multiplication medium hindered the rate of in vitro shoots proliferation (Priyadarshini et al., 2020). Along with in vitro shoot proliferation, cytokinin and auxin in combination also promote bulbet formation as supported by few studies carried out on iris species and related bulbous plants (Nower 2007; Aremu et al. 2015; Wang et al. 2018). Together with auxins, cytokinins regulates the cell division, elongation and also lead to synthesis of endogenous PGRs, hence leads to shoot regeneration and proliferation (Coenen and Lomax 1997; Gupta et al. 2020).

Keeping in view, the ornamental value of bulbous crops, the increased number of bulblets with larger size is highly desirable feature during in vitro propagation. Sucrose plays a significant role in increasing the number and size of in vitro bulblets (Kumar et al. 2005). Incorporation of sucrose at higher concentrations in the medium resulted in increase in bulblet number and also enhanced bulblet size as it leads to increased average fresh mass and diameter of in vitro bulblets confirming the previous studies (Bonnier and Van Tuyl 1997, Langens-Gerrits et al. 2003; Kumar et al. 2005). Higher number of bulblets were obtained by increasing the level of sucrose (60 and 90 g L⁻¹) for Dutch iris. Likewise, MS medium with sucrose at 90 g L⁻¹ showed significant increase in the average number of bulbs/shoot and diameter of bulbs (3.00 and 0.70 cm, respectively) compared with 30 and 60 g L⁻¹ sucrose (Nower 2007). Similar observations have been reported in other bulbous crops (Xu et al. 2008; Youssef et al. 2019). Enhanced bulb formation was observed in Dutch iris by giving cold treatment of 5°C to the cultures for four weeks (Van der Linde et al. 1987). The increase in bulblet size at high concentration of sucrose was mainly due to an increase in starch and total carbohydrates (Langens Gerrits et al. 1997; Gao et al. 2018; Hou et al. 2021). Moreover, using paclobutrazol along with high sucrose concentration had stimulation effect on the bulblet size/diameter compared with the control and sucrose alone. Presence of 25 μM paclobutrazol and 90 g L⁻¹ sucrose in the MS nutrient media resulted in significant increase in the average number of bulbs/shoot and diameter of bulbs (Nower 2007). Paclobutrazol as a growth retardant leads to accumulation of soluble carbohydrates and starch in the bulb and thus helps in increasing the bulb size (Wu et al. 2019). Bulb size and bulb formation of Lilium was significantly enhanced by the addition of paclobutrazol in Lilium plantlets (Wang et al. 1999; Thakur et al. 2006; Youssef et al., 2019; Azeri and Öztürk 2021). This increase in bulblet size by addition of paclobutrazol can be due to the shift in the partitioning of assimilates from the leaves to the storage organs and roots and increased chlorophyll and carbohydrates in all parts of seedlings (Wu et al. 2019).

In vitro rooting and acclimatization of plants is the last but most important process in the development of in vitro regeneration system for any plant species. In our results, we have found that both BAP and mT derived shoots showed better rooting response when auxin (0.5 mg L⁻¹)
IAA) was supplemented in the ½ MS medium. Similar studies in different species of Lilies have reported higher number of roots per shoot, along with increased length of roots by using auxin in MS medium (Sahoo et al. 2018; Manokari et al. 2022). However, in vitro rooting in bulblets of *Muscari azureum* on half strength MS medium supplemented with 1 mg L\(^{-1}\) IBA + 0.5 g L\(^{-1}\) activated charcoal has been reported. (Uranbey et al., 2010). Compared to BAP derived shoots, shoots from mT medium showed higher potential for in vitro rooting. In various plant species use of BA during in vitro shoot regeneration and proliferation resulted in negative effect on in vitro root formation (Werbrouck et al. 1996; Bairu et al. 2007). The better rooting efficiency of mT derived shoots may be attributed to the improved quality of shoots due to increased rate of photosynthesis (Wojtania 2010; Aremu et al. 2012). Therefore, mT is a potent cytokinin in plant tissue culture that play a multitasking role for in vitro shoot induction, multiplication and in vitro rhizogenesis in plants (Gentile et al. 2014; Gentile et al. 2017). Well rooted plantlets were acclimatized well after 4 weeks of transfer. The success of any tissue culture protocol is determined by its survival rate during hardening (Naaz et al. 2019). It is a crucial phase where in vitro raised plants has to move from a controlled protective environment to normal field conditions which can be injurious to their survival. In our study we have reported 89.11 percent survival rate of in vitro raised plants in cocopeat + sand (1:1) mixture. Similarly, best *ex vitro* survival rate (98.96%) of rooted plantlets during hardening in perlite + vermiculite (1:1) mixture was obtained in Oriental Lily (Rafiq et al. 2021).

The genetic stability of in vitro regenerated plantlet is a critical prerequisite for mass multiplication of specific genotypes (Lattoo et al. 2006). Such analysis helps in the prediction and determination of the genetic similarity between the in vitro raised clones and mother plants (Thakur et al. 2016). SCoT makers have ATG start codon in their sequence, are simple to use, cost effective, and effectively used for genetic fidelity, genetic diversity and other plant development process studies (Rai et al. 2012; Elayaraja et al. 2019; Fang-Yong and Ji-Hong 2014; Rahmani et al. 2015). In the present study the banding patterns of SCoT markers were assessed to ascertain the genetic stability of in vitro raised plants of Dutch iris to prove the production of true-to-type plants using the twin scale method. The regenerated plantlets developed did not reveal any somaclonal variation due to in vitro culture conditions thus confirming their clonal stability. Similar results have been reported in tissue culture raised plants of many other species such as Rain Lily (*Zephyranthes gran-diflora*) Dendrobium (*Dendrobium nobile* Lindl), Iris (*Iris sibirica*), Allium (*Allium sativum* L.) and Lilium (*Lilium davidii* var. unicolour Salisb) when tested for genetic similarity using molecular markers (Gangopadhyay et al. 2010; Bhattacharyya et al. 2014; Stanišić et al. 2015; Wen et al. 2020; Yang et al. 2021).

**Conclusion**

An efficient and reliable method for in vitro propagation using meta-Topolin has been optimized in *Iris × hollandica* Tub. cv. Professor Blaauw for the first time. The use of twin scale explants along with the basal plate attached were found promising for the in vitro regeneration in Dutch iris. The findings of the study revealed the superiority of mT over the commonly used cytokinin (BAP) for in vitro organogenesis in Dutch iris. The optimized photoperiod (Initial 1 week dark incubation) conditions along with 1.0 mg L\(^{-1}\) mT had a significant stimulating effect on the *ex vitro* shoot induction in Dutch iris. Syn-ergistic effect of mT with auxin (NAA) enhanced in vitro shoot proliferation and bulblet formation. Presence of high concentration of sucrose and paclobutrazol in the culture medium affected bulblet number and bulblet size positively. The shoots from the mT medium were found to be healthier and long enough, thus showed better rhizogenesis as compared to BAP derived shoots on solid ½ MS medium supplemented with auxin (IAA). The well rooted plantlets were successfully acclimatized with 89.16% survival rate under *ex vitro* conditions. Genetic fidelity evaluation using SCOT primers revealed no variation among the thirteen randomly selected in vitro raised plants of Dutch iris. Application of mT for the establishment of in vitro regeneration technology in Dutch iris will be useful for the propagation of high quality planting material of this commercial as well as other wild varieties of iris.

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**Author contributions** VV and BB conceived and planned the work. VV designed the experiments. VV, AK, P and S performed the experiments. VV and MT performed the data analysis. VV wrote the original draft of the manuscript. VV and BB supervised the experiments and edited the final version of the manuscript. All authors read and approved the final version of the manuscript.

**Data availability** The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.
Declarations

Conflict of interest  The authors declare that they have no known competing interests.

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