

Meta-Topolin mediated in vitro propagation in an ornamentally 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 high-quality planting material. In the present investigation, the significance of meta-topolin (mT), a novel aromatic cytokinin, was studied on *in vitro* morphogenesis of *Iris × hollandica* Tub. cv. Professor Blaauw (Dutch Iris). Cytokinin mT (1.0 mg L^{-1}) as a better substitute to BAP (6-Benzylaminopurine) and Kn (Kinetin), has resulted in maximum shoot induction response (86.11 per cent) and increased emergence of micro shoots (4.95 shoots/explant with average shoot length of 5.65 cm) from twin scale explants. Photoperiod also affected the shoot induction, wherein initial 1-week (w) dark incubation significantly increased shoot induction response (94.44 per cent). Both meta-topolin and initial 1w dark incubation resulted in improved quality of regenerated shoots by reducing the incidence of necrosis and preventing vitrification. The collective consequence of cytokinin-auxin (1.0 mg L^{-1} mT + 0.25 mg L^{-1} NAA) showed considerably higher number of shoots (17.53) with mean shoot length (6.90 cm) and maximum number of bulbets (2.74). Positive effect of increased sucrose concentration (90 g L^{-1}) and paclobutrazol (5 mg L^{-1}) on *in vitro* bulblet formation and bulblet size was also observed. The superiority of mT over BAP was also found during *in vitro* rhizogenesis. Shoots derived from mT medium were healthy and long enough, thus showed better rooting response (63.3 %) on $\frac{1}{2}$ MS medium + 0.5 mg L^{-1} IAA after 4 weeks. The mT raised plantlets were acclimatized in a greenhouse and showed 89.6 % survival under *ex vitro* conditions. The clonal fidelity of the *in vitro* regenerated plants was evaluated using Start Codon Targeted (SCoT) markers. Out of 36 primers, 13 primers showed clearly scorable monomorphic bands, thus displaying genetic uniformity among *in vitro* regenerated plantlets. This meta-topolin mediated protocol can be routinely used for the rapid large scale production of this valuable floriculture crop.

Introduction

Iris belongs to the Iridaceae family, known to contain several hundreds of attractive flowering plant species (Asgough et al., 2009). Iris 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 ornamentally important monocotyledonous bulbous crop developed through interspecific hybridization between *Iris tingitana* from North America and *Iris xiphium* from Spain and Portugal (Keppel, 1978). Although, it doesn't exhibit any fragrance but still due to its attractive incredible flower colors, it is rated as one of the most appreciated flower species. It has distinctive and spectacular flower arrangement, multiple colors and leaf patterns, which makes its quite popular among florist 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 slow and inefficient (Hussey, 1975; Jehan et al., 1994). In a year, through vegetative propagation, iris produce only three to five daughter bulbs per mother bulb (Shibli and Ajlouni, 2000) and the new bulbs produced are most likely susceptible to the fungal, bacterial, and iris borer. Therefore, to meet the demand of quality planting material of this commercially important cultivar, others efficient methods of propagation are required. Plant tissue culture is one of the promising alternative technique to improve the quality and quantity of plant material in Dutch iris, 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 (Fennell and Van Staden, 2004; Han et al., 2005; Rice et al., 2011; Ncube et al., 2015).

Type and concentration of PGRs used in culture medium, decides the type of *in vitro* response of the explant (Yeoman, 1986). Among PGRs, cytokinins behave as multitasking growth hormone by regulating various processes such as *in vitro* shoot organogenesis through stimulated cell divisions, *in vitro* shoot proliferation and *in vitro* rhizogenesis when used with auxin (Mala et al., 2013). As cytokinin, the use of BAP 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 meta-topolins are also gaining popularity due to their ability to induce *in vitro* organogenesis, high rate of shoot proliferation, *in vitro*, rooting and generation of quality planting material without any deformity (Strnad et al., 1997; Bairu et al., 2007; Erisen et al., 2020). The metabolism differs from benzylaminopurine (BAP) as the metabolic product (benzyladenine-9-glucoside) of BAP remains located only at the basal part of the plants, whereas the major metabolic product of mT is O-glucoside have ability to get translocated quickly to different parts of explant (Werbrouck et al., 1996). Meta-Topolin, as a prolific substitute against BAP, 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 and shoot quality induction of *in vitro* rooting and acclimatization (Lata et al., 2016).

Inspite of rapid multiplication of shoots, tissue culture of the bulbous crop also leads to *in vitro* bulblet formation upon the modification of the culture medium composition and microenvironment conditions in the culture vessels. Compared to *in vitro* shoots, 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 (Podwyszyska, 2012). Therefore, for speeding up the *in vitro* breeding process, efforts to increase *in vitro* bulblet number and size are needed. The growth and size of bulb depend upon the distribution of carbohydrates in root and shoot of a plant. The supplementation of bulblets with sucrose and growth retardants like paclobutrazol exogenously in the culture medium affects the bulb size under *in vitro* conditions (Saos et al., 2002; Ptak et al., 2014). As a known inhibitor of gibberellin biosynthesis, paclobutrazol retard shoot elongation and lead to an increase in the root-to-shoot ratio (bulb weight) by translocation of assimilates

from the leaves to the storage organs in various bulbous and tuberous species (Li et al., 2014; Yan et al., 2011; Harvey et al., 1991).

Previous studies carried out on the development of micropropagation protocol in Iris species (Van der Linde et al., 1987; Hussey, 1976; Yabuya et al., 1991; Meng et al., 2007; 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 meta-topolin for *in vitro* organogenesis in Dutch Iris. Other factors such as photoperiod, sucrose, paclobutrazol and the genetic fidelity of *in vitro* regenerants of Dutch were also studied which were not assessed in any of the previous reports. Therefore, this study aimed to develop meta-topolin mediated reliable *in vitro* propagation protocol in Dutch Iris. The genetic fidelity of the plants raised under *in vitro* conditions will be evaluated. The applicability of the developed protocol for this commercial variety can be used other varieties and wild ornamental cultivars 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).

Methods

Plant material and disinfection of bulbs

Healthy bulbs of *Iris × hollandica* Tub. cv. Professor Blaauw (Dutch Iris) were collected from the field of Floriculture, Agrotechnology division, CSIR-IHBT Palampur, India at an altitude of 1390 m above sea level with average rainfall of 2690 mm. After procurement of bulbs (4-5 cm in size) of Dutch Iris the outermost layer of tunics was removed from the bulbs and then thoroughly washed with running tap water for the removal of soil particles sticking to it. Further, bulbs were disinfected by giving a quick wash for 2-3 minutes with 0.1% (v/v) Tween-20 solution (Sigma-Aldrich, India) and followed by continuous wash under low stream running tap water for about 40 min to get rid of detergent or soap from the explant. After rinsing off bulbs properly, pre-treatment with hot water at 40 °C in water bath for about one hour was carried out. Under laminar Air flow surface sterilization of bulbs was performed by dipping them in 0.2% Bavistin (Biostadt India Ltd., India) for 20 minutes followed by rinsing with autoclaved distilled water for three to four times. After fungicide treatment, bulbs were soaked into 70% (v/v) ethanol (HiMedia, India) for 30 secs and sequentially washing with autoclaved distilled water was carried out three times. The bulbs were further decontaminated by treating them with 0.1% (w/v) HgCl₂ (HiMedia, India) solution for 5 min followed by five to six washings with autoclaved distilled water was performed to remove the any residual disinfectant.

Media and culture conditions

Throughout the study, MS basal medium (Murashige and Skoog, 1962) 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. Plant growth regulators (PGRs) such as 6-benzylaminopurine (BAP), 6-furfurylaminopurine (Kinetin/Kn), meta-Topolin (mT), 1-Naphthleneacetic acid (NAA), IAA (Indole 3-acetic acid) 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) and then poured into glass jars (*in vitro* establishment and proliferation experiment) and culture tubes (*in vitro* rooting experiment) for sterilization in autoclave at 121°C for 20 min. The culture room was maintained at 25 ± 2°C, 16/8 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.

Explants preparation and *in vitro* establishment of cultures

With the help of surgical blade, the surface sterilized bulbs were dissected longitudinally to get 6 twin-scales explants (3 cm long). Each twin scale explant joined by 5 mm of the basal plate was cultured on Murashige and Skoog (1962) medium (MS) supplemented with different concentration (0.5 to 2.0 mg L⁻¹) of BAP and Kinetin and mT to study the effect of different cytokinins on the *in vitro* shoot organogenesis. All 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. Moreover, effect of photoperiod on the *in vitro* shoot induction response was also studied by keeping the twin scale explants on the regeneration medium by giving varying photoperiods durations including (i) 16/8 h (16 h light/ 8 h Dark) photoperiod for 4 weeks (w) (ii) 1w dark followed by 16/8 h for 3w (iii) 2w dark followed by 16/8 h for 2w (iv) 3w dark followed by 16/8 h for 1w (v) complete dark for 4w

***In vitro* shoot proliferation and bulblet formation**

To confirm the synergistic effect of cytokinins and auxins on the *in vitro* shoot proliferation, shoots (3-5 cm) developed from meristematic region of basal plate were isolated and transferred on to multiplication medium comprising solid MS medium, with optimized cytokinin concentration (0.5 mg L⁻¹ BAP) and (1.0 mg L⁻¹ mT) with IAA and NAA (0.25 mg L⁻¹ and 0.5 mg L⁻¹). The shoot number proliferated per culture clump, shoot length 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 from the optimized multiplication medium were transferred to MS medium with different concentrations of sucrose (30, 60, 90 g L⁻¹) alone or in combination with growth retardants (5 mg L⁻¹ paclobutrazol) to study the effect of both sucrose and paclobutrazol on bulblet formation 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**

For *in vitro* rooting, healthy and long micro-shoots (4-5 cm) of Dutch Iris derived from BAP and mT containing multiplication medium were excised and transferred onto half-strength solid MS medium alone and in combination with auxins (IAA and NAA) at low concentration of 0.5 mg L⁻¹ and 3% (w/v) sucrose, solidified with 0.6 % (w/v) Agar- Agar (HiMedia, India). The data on percent rooting, root number, root length were recorded after 4 weeks.

For hardening, well rooted plantlets were taken out from the culture tubes and gently washed with running tap water to remove any adherent agar – agar. The rooted plantlets of Dutch iris were then transferred to plastic pots (10 cm) containing cocopeat + sand (1:1), The pots were covered with transparent polythene bags initially for two weeks for keeping high humid conditions for plants. These transplanted pots were placed under a 16/8 h (day/night) and watered with ¼ inorganic solution of MS medium every alternative day for 2 weeks. The covered plants were gradually exposed to the external environment by making holes in the poly bags and finally removed to acclimatize the plantlets to natural conditions. After 4 weeks, well acclimatized plantlets were transferred to in soil containing pots maintained in a greenhouse under natural day light conditions. The percent survival and height of *in vitro* raised plants of Dutch Iris was recorded at weekly interval until 4 weeks.

Genetic fidelity analysis of *in vitro* regenerated plantlets of *Iris × hollandica* Tub. cv. Professor Blaauw

For Genetic fidelity studies total genomic DNA was extracted from leaf tissues (100 mg) of the 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 50ng template from total genomic DNA. Go Taq® Green Master mix 2X containing 400µm dNTPs, 2x DNA polymerase, and 3Mm MgCl₂ along with 20 pmol of primer, were used for 25µl 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 10x 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 1x TAE buffer at 80v for 1-2 hours. The size of the DNA fragments was estimated by using 100bp ladder. Fragments of the DNA for each SCoT primers was visualized under UV light and photographed using gel documentation system (Molecular imager® Bio-Rad).

Experimental design and data analysis

All experiments were conducted according to completely randomized design (CRD) with three replications per treatment (30 explants per replicate). The collected data were subjected to analysis of variance (ANOVA) and the difference between means was evaluated using Duncan's Multiple Range Test (DMRT)

at the significance level $p < 0.05$ using statistical package of SPSS (Version 17.0). The results are presented in tables is untransformed numerical data (mean $\pm$ standard error).

Results

Effect of different cytokinins and photoperiod on establishment of culture and *in vitro* shoot induction

The twin scale bulb explants having attached basal plate attached cultured on MS medium with various concentrations of BAP, Kin and mT showed shoot induction within 1 to 2 w. The percentage of shoot induction response from twin scale explants varied from 0.00% (PGR-free MS medium) to $86.11 \pm 4.81\%$ (Solid MS + 1.0 mg L^{-1} mT) on different concentrations of the cytokinins tested (Table 1). After Twin scale explants on MS medium without additive and PGRs (control) did not show any shoot induction and slowly explant turned necrotic. Twin scale plants cultured upon MS medium supplemented with kinetin showed *in vitro* response Although media containing different Cytokinins, such as BAP, Kn and mT were found to be responsive for *in vitro* shoot bud induction. It was observed that meristematic cells of the basal plate of scales of bulb get activated for organogenesis by Cytokinins treatment. But among different cytokinins used, mT was found to be best for *in vitro* shoot induction from twin scale explants, thus explains its advantage over using BAP and Kn. The shoot induction response and number of shoots induced from twin scale explants was highest ($86.11 \pm 4.81\%$) on MS medium containing 1.0 mg L^{-1} mT, (Table 1) with maximum number of micro-shoots (4.95 ± 0.05). The maximum shoot length (5.65 ± 0.13 cm) was also observed on the same medium (Table 1 and Fig. 1). The effect of photoperiod was also studied on *in vitro* shoot induction on the optimized regeneration medium (MS supplemented with mT 1.0 mg L^{-1}). In this experiment, 1-week dark incubation prior to 16 h/8h light conditions for 3 weeks were found to be most appropriate for *in vitro* shoot induction as compared to normal 16h/8h photoperiod and initial dark incubation for 2, 3 and 4 weeks (Fig. 2). Dark treatment for 1 week on the optimized regeneration medium treatment in 94.44 ± 1.38 per cent shoot induction with 4.76 ± 0.39 average number of shoots per explant (Fig. 3). Moreover, initial short dark incubation of 1 w reduced the incidence of necrosis by overcoming phenolic exudation from the explant. Longer dark incubation periods of 4 weeks resulted in reduced shoot induction response ($45.83 \pm 4.16\%$) with 2.36 ± 0.31 shoot number. Moreover, shoots obtained from longer dark incubated cultures turned yellow.

Table 1

Effect of different concentrations of the cytokinins such as BAP, Kn, and mT on *in vitro* shoot induction from twin scale bulb explants of *Iris × hollandica* Tub. cv. Professor Blaauw after 4 weeks of culture

Cytokinins (mg L ⁻¹)	Shoot induction response (%)	Number of shoots (mean ± SE)	Shoot length (cm) (mean ± SE)
Control	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
BAP 0.5	69.44 ± 4.81 ^e	1.50 ± 0.50 ^b	2.90 ± 0.04 ^f
BAP 1.0	47.22 ± 9.62 ^c	1.08 ± 0.02 ^b	2.38 ± 0.19 ^e
BAP 1.5	66.67 ± 8.33 ^{de}	1.73 ± 0.12 ^b	2.89 ± 0.07 ^f
BAP 2.0	55.56 ± 4.81 ^{cd}	1.48 ± 0.27 ^b	2.04 ± 0.04 ^d
Kn 0.5	27.78 ± 4.11 ^b	1.20 ± 0.15 ^b	1.27 ± 0.10 ^{bc}
Kn 1.0	22.22 ± 4.23 ^b	1.07 ± 0.07 ^b	1.17 ± 0.03 ^{bc}
Kn 1.5	25.00 ± 14.43 ^b	1.23 ± 0.15 ^b	1.37 ± 0.14 ^c
Kn 2.0	16.67 ± 8.33 ^b	1.05 ± 0.05 ^b	0.94 ± 0.07 ^b
mT 0.5	77.78 ± 9.62 ^{ef}	4.27 ± 0.37 ^d	4.73 ± 0.23 ^h
mT 1.0	86.11 ± 4.81 ^f	4.95 ± 0.05 ^e	5.65 ± 0.13 ^j
mT 1.5	80.56 ± 4.80 ^{ef}	3.12 ± 0.06 ^c	5.09 ± 0.09 ⁱ
mT 2.0	72.22 ± 9.61 ^{ef}	3.15 ± 0.21 ^c	4.11 ± 0.12 ^g
Values represent mean ± SE of 15 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (p = 0.05) using Duncan's Multiple Range Test			

Interactive 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, shoots obtained from twin scale explants will be subcultured on MS medium supplemented with optimized 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 effect of Cytokinins (BAP and mT) with auxins (NAA and IAA) on *in vitro* proliferation was also observed. 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 regenerated on the combination of cytokinin and auxin particularly, 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, IAA) were significantly higher than that of the number of shoots observed on the BAP alone or BAP with auxins. and. Results presented in Table 2 and Fig. 4 (A-D) 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 resulted in the maximum number of shoots (17.53 ± 0.29), highest shoot length ($6.90 \pm 0.08 \text{ cm}$) and highest number of bulblets (2.74 ± 0.08)

Table 2
Synergistic effect of optimized cytokinins (BAP and mT) with auxins (IAA and NAA) on *in vitro* proliferation of *Iris x hollandica* Tub. cv. Professor Blaauw after 4 weeks of culture

BAP (mg L^{-1})	mT (mg L^{-1})	IAA (mg L^{-1})	NAA (mg L^{-1})	Number of shoots (mean $\pm$ SE)	Shoot length (cm) (mean $\pm$ SE)	Number of bulblets (mean $\pm$ SE)
0.5	0.0	0.0	0.0	5.22 ± 0.04^a	4.11 ± 0.39^a	0.97 ± 0.03^a
0.5	0.0	0.25	0.0	5.83 ± 0.42^a	3.25 ± 0.19^a	1.96 ± 0.03^{cd}
0.5	0.0	0.5	0.0	7.33 ± 0.24^b	4.67 ± 0.09^{bc}	1.35 ± 0.05^b
0.5	0.0	0.0	0.25	8.19 ± 0.19^{bc}	5.34 ± 0.22^{bc}	1.90 ± 0.01^c
0.5	0.0	0.0	0.5	8.99 ± 0.01^c	4.87 ± 0.15^c	1.49 ± 0.12^b
0.0	1.0	0.0	0.0	9.08 ± 0.08^c	4.65 ± 0.14^b	2.10 ± 0.05^{cde}
0.0	1.0	0.25	0.0	11.1 ± 0.60^d	5.83 ± 0.13^d	1.93 ± 0.07^{cd}
0.0	1.0	0.5	0.0	13.50 ± 0.76^e	5.89 ± 0.14^d	2.18 ± 0.10^{de}
0.0	1.0	0.0	0.25	17.53 ± 0.29^f	6.90 ± 0.08^e	2.74 ± 0.08^f
0.0	1.0	0.0	0.5	15.74 ± 0.53^g	5.98 ± 0.03^d	2.28 ± 0.14^e
Values represent mean $\pm$ SE of 15 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different ($p = 0.05$) using Duncan's Multiple Range Test						

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

This experiment was conducted to study the effect of sucrose at different concentration (30, 60, 90, g L⁻¹) alone and in combination with growth inhibitor 5 mg/l paclobutrazol) on *in vitro* bulblet formation in Dutch Iris. Data presented in Fig. 5A 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 ± 0.08 and 3.91 ± 0.04 , respectively) as compared to MS medium with 30 L⁻¹ sucrose (2.52 ± 0.04). 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 sucrose at concentration 30 g L⁻¹, 60 L⁻¹ and 90 g L⁻¹ has showed slight reduction in number of bulblet formation (2.06 ± 0.15 , 3.36 ± 0.03 , 3.44 ± 0.13) respectively. But the presence of Paclobutrazol has increased bulblet diameter significantly at higher sucrose in MS medium (60 and 90 g L⁻¹). The maximum bulblet diameter (2.28 ± 0.04 cm) was observed in MS medium supplemented with 90 g L⁻¹ +5 mg L⁻¹ Paclobutrazol (Fig. 4G & 5B).

In vitro rhizogenesis and acclimatization of in vitro raised plantlets

In this experiment *in vitro* rooting of the BA and mT derived micro-shoots of *Iris × hollandica* Tub. cv. Professor Blaauw was compared on ½ MS medium with (0.5 mg L⁻¹ IAA) or without auxins. Addition of auxin in ½ MS medium has augmented *in vitro* rooting response from both BA and mT derived shoots. mT derived micro-shoots, showed significant increase in rooting response on both ½ MS and ½ MS medium supplemented with 0.5 mg L⁻¹ IAA as compared to BA derived shoots. The highest per cent rooting (63.83 ± 0.93) and highest number of roots (3.42 ± 0.08) with root length (4.48 ± 0.14 cm) was recorded from mT derived shoots on ½ MS medium supplemented with 0.5 mg L⁻¹ IAA after 4 weeks of transfer (Table 3 & Fig. 6A-D). Whereas, in the case of BA-derived microshoots, 35.83 ± 0.83 per cent *in vitro* rooting with maximum number of roots (2.15 ± 0.08) and lengths (2.46 ± 0.05) per microshoot 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 BA. After 4 weeks, well rooted *in vitro*-raised healthy plantlets/bulblets were taken out from the culture tubes and transferred to plastic pots with autoclaved cocopeat and Sand (1:1) mixture and All the plantlets were covered with perforated plastic bags and kept inside growth room with high humidity (80–90%) and $25 \pm 2^\circ\text{C}$ temperature conditions. After two weeks, the polybags were gradually removed to for the gradual acclimatization of the plantlets. The growth and survival of plants was observed at weekly intervals. The maximum per cent survival rate of plants of *Iris × hollandica* cv Professor Blauw in the greenhouse after 4 weeks was 89.16 ± 0.58 with 15.24 ± 0.08 cm long plants (Fig. 6E-F & Fig. 7). The hardened plantlets were shifted to pots containing normal garden soil

Table 3

Comparative analysis of *in vitro* response of rooting of the BA and mT derived micro-shoots of *Iris × hollandica* Tub. cv. Professor Blaauw after 4 weeks of culture

	Medium	Percent Rooting (mean ± SE)	No. of roots per shoot (mean ± SE)	Root length (cm) (mean ± SE)
BAP*	1/2MS	23.33 ± 1.67 ^a	0.97 ± 0.03 ^a	1.13 ± 0.12 ^a
	1/2 MS + 0.5 mg L ⁻¹ IAA	35.83 ± 0.83 ^b	2.15 ± 0.08 ^b	2.46 ± 0.05 ^b
mT*	1/2MS	57.08 ± 0.42 ^c	2.63 ± 0.16 ^c	3.74 ± 0.05 ^c
	1/2 MS + 0.5 mg L ⁻¹ IAA	63.83 ± 0.93 ^d	3.42 ± 0.08 ^d	4.48 ± 0.14 ^d
*The micro-shoots subjected to <i>in vitro</i> rooting were obtained from MS medium supplemented with BAP and mT respectively. Values represented means ± SE. Means followed by the same letter within column are not significantly different (<i>P</i> = 0.05) using Duncan's multiple range test				

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. 8). A total of 47 bands with an average of 4.06 bands per SCOT primer, among regenerants were scored and 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. 8A, B C respectively.

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	ACGACATGGCGACCAACG	4
2	Scot 15	ACGACATGGCGACCGCGA	5
3	Scot 19	ACCATGGCTACCACCGGC	2
4	Scot 21	ACGACATGGCGACCCACA	5
5	Scot 22	AACCATGGCTACCACCAC	3
6	Scot 24	CACCATGGCTACCACCAT	4
7	Scot 25	ACCATGGCTACCACCGGG	5
8	Scot 27	ACCATGGCTACCACCGTG	4
10	Scot 30	CCATGGCTACCACCGGCG	3
11	Scot 31	CCATGGCTACCACCGCAC	3
12	Scot 32	CCATGGCTACCACCGCAG	4
13	Scot 35	CATGGCTACCACCGGCCC	5
Total No. of Bands 47			

Discussion

Generally, *in vitro* regeneration systems are significantly influenced by various factors such as genotype of the explant used, media composition (particularly PGRs) and photoperiod (Gantait et al., 2010; García-Forte et al., 2020). Therefore, for the development of efficient regeneration system for a particular species, optimized culture medium and culture conditions are of prime requirement (Barandiaran et al., 1999). Although few 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; Meng et al., 2007; Zhu et al. 2015; Wang et al., 2018). But to our knowledge, there is as yet no consistent, efficient and reliable regeneration system for Dutch Iris. Here, we reported the development of efficient *in vitro* propagation protocol using twin scale explants of *Iris × hollandica* Tub. cv. Professor Blaauw by optimizing various physiochemical factors including PGRs (Cytokinins and auxins), photoperiod, additives such as sucrose and growth inhibitor (Paclobutrazol).

Cytokinins plays crucial role for *de novo* shoot organogenesis in any plant species, as the use of optimal and effective cytokinins in tissue culture medium determines the morphogenetic ability (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 *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 et al. 2019). In contrast to our results, maximum 12 shoots per twin scale were obtained on MS medium augmented with 2.0 mg L^{-1} BAP in *Crinum malabaricum* (Priyadharshini et al., 2020). Being a potent cytokinin, BAP is most preferred for *de novo* organogenesis from mature explants due to its ability to metabolize in cells and activation of meristematic centres for shoot bud induction (Gupta et al., 2014). Despite of its advantages in tissue culture, it 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). 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 16h/8h photoperiod conditions for 4 weeks. 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 *Hyacinthus orientalis* and *Lilium longiflorum* (Kim et al., 1981 and Kumar et al., 2006). Conversely, maximum number of bulblets/leaves were recorded from the cultures kept in 16/8 h light/dark in *Narcissus tazetta L.* was reported (Khonakdari et al., 2021). 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). The distinct and better shoot induction response of bulb scale explants of Dutch Iris under initial 1-week darkness over the other photoperiod conditions may be attributed to the inhibition of exudation of phenolic from the explant. This suggests that continuous light increases the chances of explant browning due to phenolic exudation and thus reduces the shoot induction response *in vitro* (Naidoo et al., 2017). Previous *in vitro* regeneration studies on bulbous crops have revealed that *in vitro* organogenesis from twin-scales can be achieved under different photoperiodic conditions (light and dark conditions) (Rice et al. 2011). This suggest that requirement of different photoperiod on *in vitro* morphogenetic response is species and genotype specific.

As discussed, cytokinins play significant role in *in vitro* organogenesis but the choice of cytokinins and cytokinin-auxin combination is a determining factor for enhanced *in vitro* shoot proliferation (Naidoo et al., 2017). In our study, 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, enhanced *in vitro* shoot multiplication was recorded in different species of *Lilium* such as *Lilium longiflorum* 'Georgia' (Han et al., 2004) and *Lilium orientalis* (Youseff et al., 2019) on the use of cytokinin with lower dose of auxin in the culture medium. 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 (Aremu et al., 2015; Wang et al., 2018). Studies in different plant species such as *Mitragyna parvifolia* (Patel et al. 2020), *Maytenus emarginata* (Shekhawat et al., 2021) have also reported interactive effect of cytokinin or mT and auxin on *in vitro* shoot proliferation. 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). Therefore, synergism of mT with NAA had a good effect on shoot proliferation and improving shoot rate multiplication (Bogaert et al., 2006 and Ferguson and Beveridge 2009). 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).

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*. Similar observations have been reported in other bulbous crops (Xu et al., 2008 and Youssef et al., 2019). 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. Plant growth retardants such as trizole and its derivatives, reduce shoot elongation by inhibiting cytochrome P-450-mediated oxidative demethylation in the gibberellin biosynthetic pathway (Kamoutsis et al. 1999). 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 and Thakur et al., 2006; Youseff et al., 2019; Azeri et al., 2021). This increase in bulblet size by addition of paclobutrazol can be due to the to a 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 mgL⁻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 increased length of roots by using auxin in MS medium (Sahoo et al., 2018; Taha et al., 2018).

However, Uranbey et al., 2010 has reported *in vitro* rooting in bulbets of *Muscari azureum* on half strength MS medium supplemented with 1mg/l IBA + 0.5 g/l activated charcoal. Compared to BA 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 for/mation (Werbrouck et al., 1995; Bairu et al., 2008). 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 and 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 2017). Well rooted plantlets were acclimatized well after 4 weeks of transfer. The success of any tissue culture protocol is dermined 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 per cent survival rate of *in vitro* rasied 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 Lilium Hybrid Cv. 'Ravenna' (Rafiq et al., 2021).

The genetic stability of *in vitro* regenerated plantlet is a critical prerequisite for mass multiplication of specific genotypes including putative transformants (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). The SCoT markers have ATG start codon in their sequence, and used in genetic diversity and DNA fingerprinting studies for detection of genetic polymorphism in plant genes (Collard and Mackill 2009). SCoT makers 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 epigenetic 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 *Zephyranthes grandiflora* (Gangopadhyay et al., 2010), *Dendrobium nobile* Lindl (Bhattacharyya et al., 2014); *Iris sibirica* (Stanišić et al., 2015), *Allium sativum* L (Wen et al., 2020) and *Lilium davidii* var. unicolour Salisb (Yang et al., 2021) when tested for genetic similarity using molecular markers

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 (BA) for *in vitro* organogenesis in Dutch Iris. The optimized photoperiod (Initial 1week dark incubation) conditions along with 1.0 mg L⁻¹ mT had a significant stimulating effect on the *in vitro* shoot organogenesis in Dutch iris. Synergistic

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 mT medium were found to be healthier and long enough, thus showed better rhizogenesis compared to BA derived shoots on soild ½ MS medium supplemented with auxin (IAA). The well rooted plantlets were successfully acclimatized with 89.16 per cent 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 *Iris × hollandica* Tub. cv. Professor Blaauw. 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 wild varieties of Iris.

Declarations

Authors' Contributions

VV and BB conceived and planned the work. VV designed the experiments. VV, AK, P and SC 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.

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Data availability

The data supporting the findings of this study are available from the corresponding authors, upon request.

Conflict of interest

The authors declare that they have no known competing interests.

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Figures



Figure 1

In vitro establishment and shoot regeneration in *Iris x hollandica* Tub. cv. Professor Blaauw using twin bulb scale explants

A) Healthy bulbs with tunics, B) Surface sterilized bulbs used for preparing explants, C) Sterilized twin-scale explants, D-F) *In vitro* establishment from twin scale explants on the MS medium with 0.5 mg·L⁻¹ BAP after 1, 2 and 4 weeks respectively, G-I) *In vitro* establishment from twin scale explants on the MS medium with 1.0 mg L⁻¹ mT after 1, 2 and 4 weeks respectively.

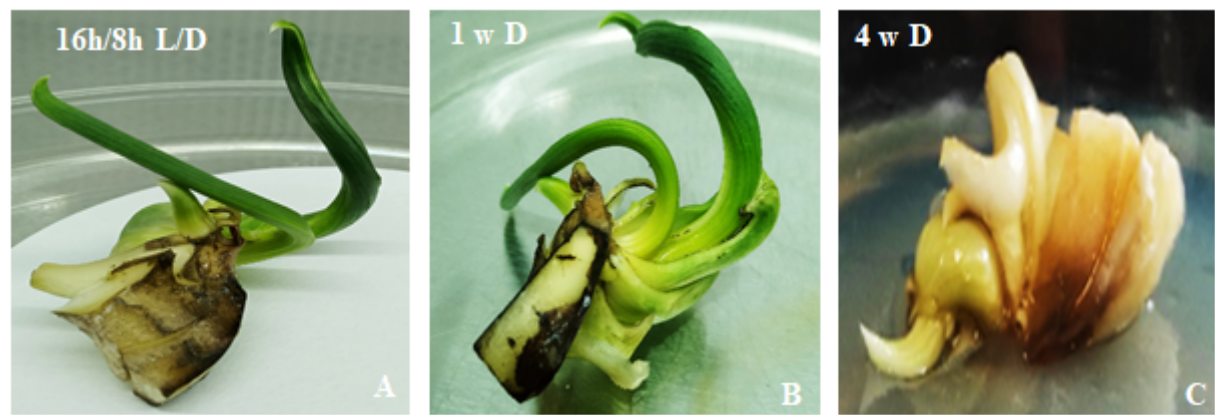


Figure 2

Representative photographs of induction of *in vitro* shoots from twin scale explant of *Iris x hollandica* Tub. cv. Professor Blaauw on the optimized regeneration medium (MS + 1.0 mg L⁻¹ mT) under A) 16h/8h photoperiod for 4 weeks, B) 1 week Dark incubation followed by 3 weeks of 16h/8h photoperiod, C) under dark conditions for 4 weeks.

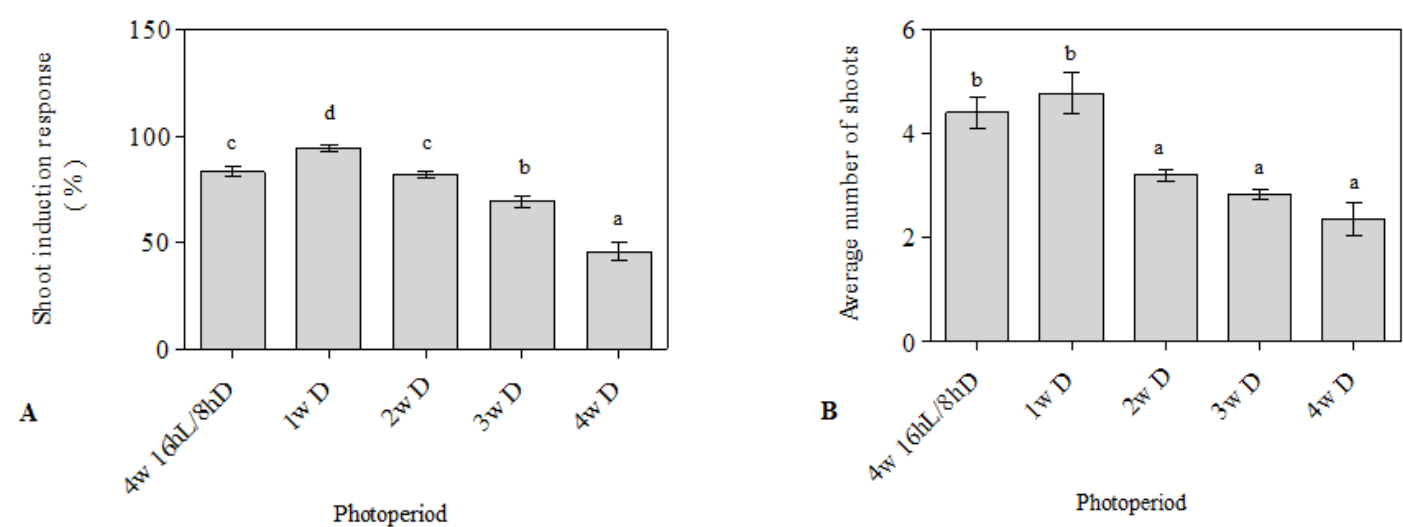


Figure 3

Effect of photoperiod on the A) shoot induction response, and B) average number of shoots formed from the twin scale explants of *Iris x hollandica* Tub. cv. Professor Blaauw after 4 weeks of culture on the solid MS medium + 1.0 mg L⁻¹ mT

Photoperiod: (i) 16/8 h for 4 weeks (ii) 1w dark followed by 16/8 h for 3 w (iii) 2 w D followed by 16/8 h for 2 w (iv) 3 w dark followed by 16/8 h for 1 w (v) complete dark for 4 w

*Means followed by the same letter are not significantly different ($p = 0.05$) using Duncan's Multiple Range Test



Figure 4

***In vitro* proliferation of micro-shoots and *in vitro* bulblet formation in *Iris x hollandica* Tub. cv. Professor Blaauw**

A-C) Multiple shoot induction on MS medium supplemented with 1.0 mg·L⁻¹ mT + 0.25 mg·L⁻¹ NAA after 1 week, 2 weeks, 3 weeks and 4 weeks of culture respectively E-F) *In vitro* bulblet formation on MS medium supplemented with 60 g L⁻¹ and 90 g L⁻¹ Sucrose respectively G) *In vitro* bulblet formed on MS medium supplemented with 90 g L⁻¹ Sucrose + 5 mg L⁻¹ Paclobutrazol (Bar 2 cm)

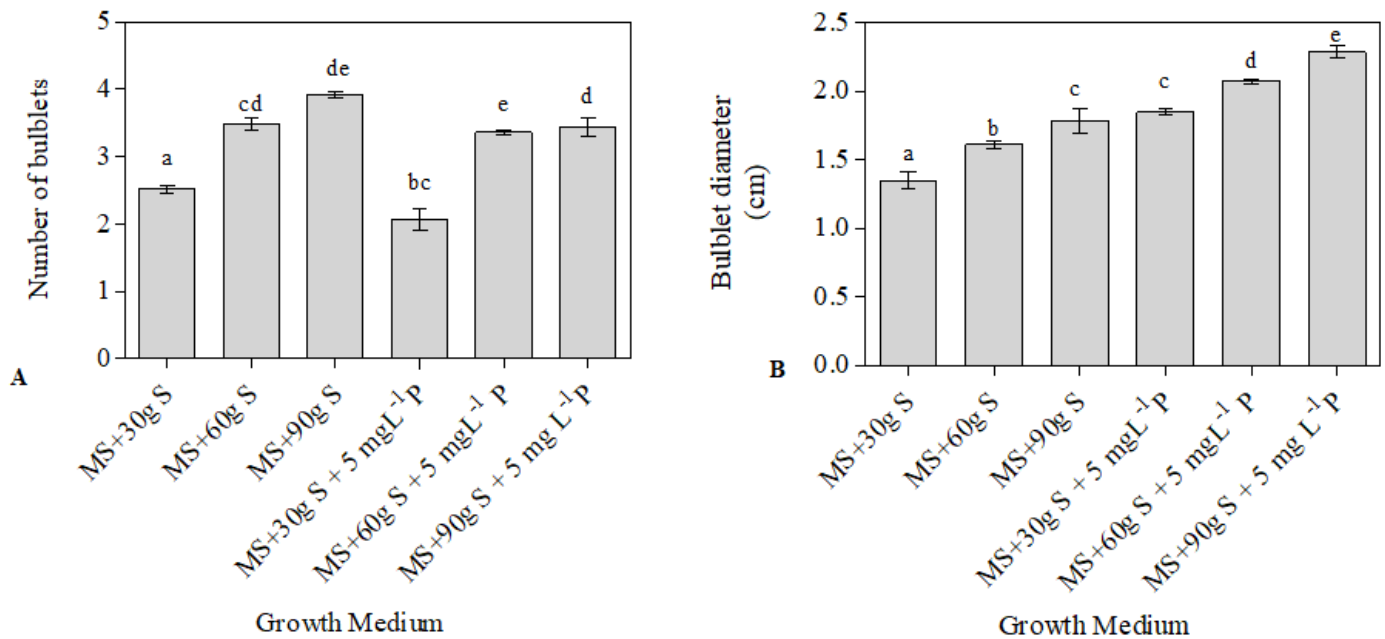


Figure 5

Effect of different concentrations of sucrose alone and in combination with paclobutrazol on the (A) number of *in vitro* bulblets and (B) bulblet diameter of *Iris x hollandica* Tub. cv. Professor Blaauw after 4 weeks of culture

*Means followed by the same letter are not significantly different ($p = 0.05$) using Duncan's Multiple Range Test

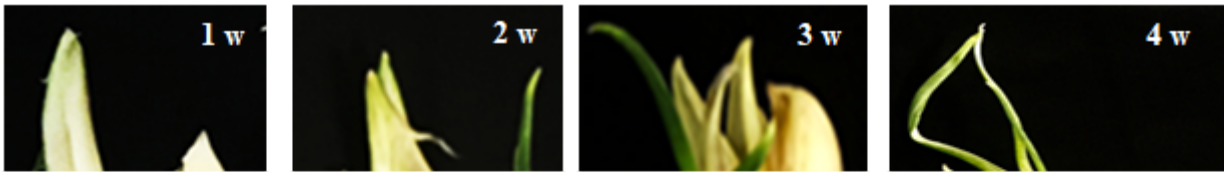


Figure 6

***In vitro* rooting of micro-shoots and acclimatization of *in vitro* plantlets of *Iris × hollandica* Tub. cv. Professor Blaauw**

A-D) *In vitro* root induction on ½ MS +0.5 mg L⁻¹ IAA at weekly intervals (1w, 2w, 3w, 4w), E) Hardening of *in vitro* plantlets in plastic pots at weekly intervals (1w, 2w, 3w), F) Acclimatized *in vitro* plants after 4 weeks of culture

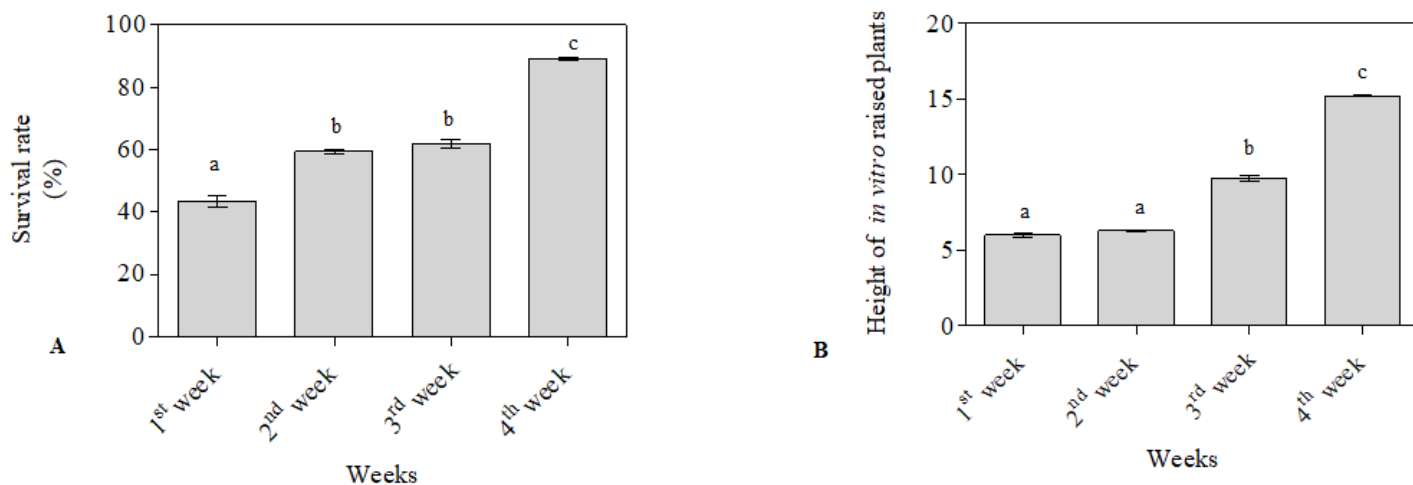


Figure 7

Effect of hardening on A) survival rate B) height of *in vitro* raised plants of *Iris x hollandica* Tub. cv. Professor Blaauw at weekly interval

*Means followed by the same letter are not significantly different ($p = 0.05$) using Duncan's Multiple Range Test

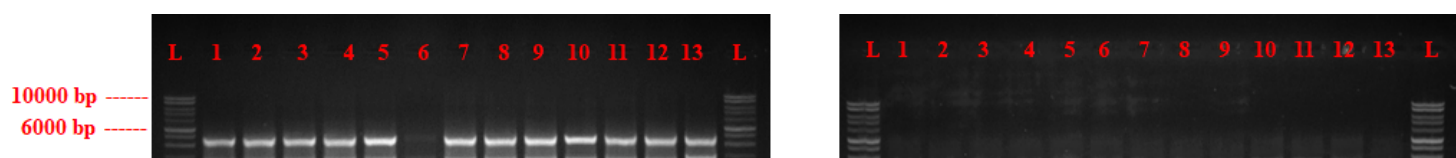


Figure 8

SCOT banding profile among *in vitro* regenerants of *Iris x hollandica* Tub. cv. Professor Blaauw. A) SCOT Primer 15; B) Primer SCOT-21; C) Primer SCOT-35 where L = ladder, 1–13 = *in vitro* regenerants