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Optimization of plantlet regeneration from leaf base derived callus of Japanese Iris (*Iris ensata* Thunb.): An ornamental medicinal plant

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Abstract
Leaf base explants of *Iris ensata* were cultured in MS basal medium containing different plant growth hormones. MS supplemented with 2,4-D (1.0 mg/l) and TDZ 0.1 mg/l) showed earlier callus initiation (25.30 days) in greater percentage of explants [96.67% (79.69%)] and produced higher callus weight at both 15 (0.373g) and 30 (2.961g) days after culture. Regenerated calli when cultured in MS + GA_3 (2.0 mg/l) + BAP (1.0 mg/l) recorded earlier regeneration (29.20 days) with greater percentage of explants [99.70% (86.97%)], greater micro shoot length (8.64 cm) and leaf length (7.00 cm); while MS+GA_3 (2.0 mg/l) + BAP (2.0 mg/l) produced maximum number of micro shoots per culture (16.30) with broadest leaf (1.32 cm). MS supplemented with 2.0 mg/l BAP yielded maximum number of leaves per micro shoot (11.70). *In vitro* regenerated micro shoots when cultured in MS medium containing IBA (1.0 mg/l) + NAA (1.0 mg/l) reported earlier root initiation (26.07 days) with higher number of roots per plant (9.07) having greater length (9.32 cm). Percent of rooted micro shoots were found highest [99.62% (86.51%)] when tried with MS + IBA (1.5 mg/l) + NAA (1 mg/l). Diameter (1.019 mm) of regenerated *in vitro* roots was found higher with MS + IBA (2.0 mg/l) + NAA (1.0 mg/l). A mixture containing equal proportion of vermiculite and vermicompost ($^{v}/_{v}$) resulted greater survivability [86.85% (68.69%)] in shorter duration (12.43 days) during subsequent acclimatization of *in vitro* regenerated plantlets of Japanese Iris. Culture of explants in hormone free MS basal medium showed least response in all growth and development aspects *in vitro*. Least percent of acclimatized plantlets [55.42% (48.15%)] was obtained from the hardening medium containing equal proportion of garden soil and vermicompost.

Keywords: Japanese Iris, *in vitro* propagation, plant growth hormones

Introduction
The *Iris* sp. belonging to the plant family Iridaceae, comprises of approximately 300 species which are widely distributed worldwide (Kassak, 2014) [7]. Irises are mainly used as the ornamental landscaping and potted plants due to their large showy colorful flowers. In Manipur, more than five species of iris are found grown wildly, out of which two species *viz*., *Iris laevigata* (endemic to the state) and *Iris sanguinea* are grown in the marshy areas of Imphal Valley. The Iris flowers are offered to God during Cheiraoba (*Meitei* New Year) in Manipur. Traditionally, the flowers are used for making dyes and the rhizomes are used as brain coolant and in hysteria. Similarly, *Iris ensata* is also used in the same manner by the people of Manipur. Many other Iris species were also known for their promising medicinal value used in treatment against a wide spectrum of diseases in many parts of the world. The isolation of variety chemical compounds including quinones, triterpenoids, flavonoids, isoflavonoids and stilbene glycosides have been reported from different intensive phytochemical investigations of various iris species (Purev *et al*., 2022) [13]. Wagay and Jain 2018 [19], on phytochemical screening of the extract of *Iris ensata* showed the presence of some common compounds like phenols, terpenoids, flavonoids, carbohydrate etc. that possess antimicrobial potential against antibacterial and antifungal strain. The genus is native to Japan, China, Korea and Russia. Like other ornamental monocotyledonous species with bulbs or rhizomes, Irises are generally propagated vegetatively through splitting of rhizomes or bulbs, where their propagation rate is very low (Jehan *et al*., 1994) [6]. Moreover, the division of rhizomes in perennial iris is desirable only after three years (Anon., 2010) [1].

Propagation through seeds cannot keep genetic uniformity, since the species is an outcrossing one (Yabuya *et al*., 1991) [20]. As well as vegetative propagation through splitting of rhizomes, the rate of propagation is very slow in irises. Therefore, micropropagation might be the alternative method of choice (Jehan *et al*., 1994) [6] for rapid generation of a large scale of disease-free, true-to-type, uniform, elite and quality planting material for this potential ornamental and medicinal value iris species. The feasibility of *in vitro* plant regeneration of Iris was first demonstrated by Fujino *et al*. (4). Since then, researchers have cultured callus of many species of Iris, using explant sources such as young inflorescence, floral parts, mature embryo (Yabuya *et al*., 1991) [20], shoot apex (Radojevic and Subotic, 1992) [14], leaf base and rhizome apex (Jehan *et al*., 1994) [6]. Various *Iris* species have been propagated through organogenesis or somatic embryogenesis, using explants from the leaf base (Shibli and Ajlouni, 2000 [15], mature zygotic embryos (Boltenkov *et al*., 2004) [2], ovary sections (Laublin and Cappadocia, 1992 [8], and root sections (Laublin *et al*., 1991) [9]. However, there is no such research report available about the ideal hormonal combination in successful *in vitro* regeneration of iris sp. Hence, the present research work has been attempted to develop efficient protocols for micropropagation of Japanese Iris (*Iris ensata* Thunb.) through standardization of hormonal combination in aseptic culture medium indicating the morphogenetic potential of calli in *in vitro* morphogenesis of Japanese Iris using leaf base as explants.

Materials and Methods

The experiment was conducted for two consecutive years at plant tissue culture laboratory, Department of Floriculture, Medicinal and Aromatic Plants, UBKV, Cooch Behar during the year 2016-2018. Plants of Japanese Iris were brought from Kombirei Garden, Manipur and were maintained in the protected structure of the Department.

Explant

The explants of Japanese Iris obtained from mature plants, exhibited a great source of contamination. Young immature, white colour leaf-base explants was carefully washed under running tap water followed by washings in tap water containing two to three drops of Tween-20 and again by running tap water for about half an hour. These were then surface sterilized with 70% absolute alcohol (20-30 seconds) followed by 0.1% mercuric chloride ($HgCl_2$) for 2 minutes and subsequently washed for 4-5 times with sterilized double distilled water to remove the traces of $HgCl_2$. These explants were then ready for use.

Transfer area and maintenance of aseptic condition

All the aseptic manipulations such as surface disinfection of the explants, preparation and inoculation of explants and subsequent sub-culturing were carried out in the laminar air flow cabinet. The working table of laminar air flow cabinet and spirit lamp were sterilized by swabbing with absolute alcohol. All the required materials like culture media, spirit lamp, flaming apparatus, glassware etc. were transferred onto the clean laminar air flow. The UV light was switched on for half an hour to achieve aseptic environment inside the cabinet before working in the lamina air flow cabinet.

Subculture: Callus (Fig. 1) and microshoots (Fig. 4) formed in the culture tubes/slant tubes were taken out after four weeks of inoculation. The shoots were separated by dissecting them in the sterile environment of laminar air flow cabinet with sterile dissecting needle and forceps. They were again placed in the respective tubes and jam bottles containing fresh media.

Callus induction and growth

Young immature leaf base sections of Japanese Iris were cultured on hormone free MS basal medium, MS + four level of 2, 4-D (1.0, 2.0, 3.0, 4.0 mg/l) alone and MS + each four level of 2, 4-D in combination with two level of TDZ (0.1 & 0.2 mg/l) were used for callus induction. Visual observations like number of days required for induction of callus, percent explant produced callus, colour, shape and texture of callus were recorded periodically. Further for culture regeneration the explants were subculture on hormone free MS basal medium, MS + each three level of GA_3 and BAP (0.5, 1.0, 2.0 mg/l) alone and MS + two level of GA_3 (2.0 & 3.0 mg/l) in combination with three level of BAP (1.0, 2.0, 3.0 mg/l).

Rooting

The micro shoots of more than 3.0-5.0 cm in height were taken out and placed/ subculture in the tubes containing fresh rooting media i.e. hormone free MS basal medium, MS + six level of IBA (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/l) alone and MS + each six level of IBA in combination with one level of NAA (1.0 mg/l) for *in vitro* roots development (Fig 6).

Acclimatization

Young rooted plantlets of Japanese Iris (Fig. 6) were taken out of the jam bottles and test tubes, washed with distilled water and planted in small pots containing different hardening media *viz*., Garden soil, sand, vermiculite and vermicompost in different combinations**.** The plantlets were given nutrient solution (starter solution containing themacro and micro nutrient stock solutions of Basal MS medium used for micropropagation) and watered regularly in the laboratory for 4 days. Then they were transferred to greenhouse for further acclimatization (Fig. 7).

The experiment was laid out under completely randomized design (CRD) consisting of thirteen number of treatments each for callus induction, plantlets regeneration, roots induction and five number for plantlets acclimatization. Fifteen cultures each were used per replications for plant caulogenesis, organogenesis, rhizogenesis and also for plantlet acclimatization. Each treatment was replicated thrice for *in vitro* plant regeneration and 5 replications were given in case of plantlets acclimatization. Data observation were made for following parameters *viz*., visual recorded on colour, shape and texture of callus; Percentage of callusing (%); Days to callus initiation (days); Weight of callus (g); Days required for shoot initiation; Regeneration percent (%); Number of micro shoots per culture; Length of micro shoot (cm); Number of leaves per micro shoot; *in-vitro* leaf length (cm); Leaf width (cm); Days required for *in-vitro* root initiation; Percentage of micro shoots rooted (%); Number of roots per micro shoot, Length of root (cm); Diameter of root (mm); Days required for acclimatization and Hardening Percentage (%).

All experiments were repeated two times. The percentile data of the experiments were assumed and subjected to square root transformations.

Statistical analysis

The data generated were analyzed by Fisher's analysis of variance (ANOVA) technique at 5 percent level of significance.

Results and Discussion

Callusing intensity and callus morphology of Japanese Iris

were recorded through visual appearance (Table 1). Best callus induction (C+++) were found when the leaf base explants were cultured in the MS basal medium supplemented with 2,4-D (1.0-2.0 mg/l) and TDZ (0.1-0.2 mg/l) producing either waxy yellowish friable to yellowish compact or greenish yellow compact calli. Callusing intensity was also reported better in the MS medium fortified with 2,4-D (1.0 mg/l) that generated yellowish nodular calli. A high concentration of 2,4-D (3.0 to 4.0 mg/l) in the MS basal medium produced whitish friable to brownish friable calli. The least callusing intensity (C+) were observed with the high concentration of 2,4-D (4.0 mg/l) in addition with TDZ (0.1 to 0.2 mg/l) producing minute yellowish friable to yellowish brown calli. Inclusion of TDZ in the callusing medium along with low or moderate concentrations of 2,4-D increased the intensity of callusing.

The data depicted in Table 2 showed that culture of Japanese Iris leaf base explants in MS basal medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l TDZ recorded the highest percentage (79.69%) of callusing explants at least number of days (25.30) to initiate and maximum weight of callus at 15 (0.373 g) and 30 (2.961g) days after initiation of callus (Fig. 2). MS basal medium devoid of any plant hormones recorded the least callusing percentage (11.33%), took a greater number of days (119.40) to initiate callus and lowest calli weights at 15 (0.023g) and 30 days (0.117g) after callus initiation. Use of TDZ in minute amount promotes the synthesis and accumulation of purines (Capelle *et al*., 1983) [4] and also alters cytokinin metabolism (Mok *et al*., 1987) [11]. Zumbakiene *et al*. 2005 [21] observed better callus proliferation in *Phalaenopsis* hybrids when cultured in MS basal medium supplemented with 1-20 mg/l 2,4-D and 1 mg/l TDZ.

Results from Table 3 showed that addition of GA_3 (2.0 mg/l) and BAP (1.0 mg/l) in MS medium represented earlier (29.20 days) regeneration of Japanese Iris from callus with a higher percentage (86.97%). The same culture medium produced tallest micro shoots (8.64 cm), with maximum leaf length (7.00 cm) *in vitro*. However, MS basal medium supplemented with 2.0 mg/l GA_3 + 2.0 mg/l BAP produced maximum number of micro shoots per culture (16.30) and broadest leaf (1.32 cm); while MS fortified with 2.0 mg/l BAP yielded maximum number of leaves per micro shoot (11.70). In all the attributes of explants, culture in hormone free basal MS medium reflected the least performance. The higher *in vitro* growth and development of micro shoots occurred due to the growth promotive action of the plant growth regulators used in the culture media as well as absence of apical dominance. Both gibberellins and cytokinin are having active role in growth and development of meristematic tissues (Shimizu *et al*., 1996) [16]. A combination of gibberellins and cytokinin was also found effective during micro shoot growth and development of Japanese Iris was also observed by (Shimizu *et al*., 1996) [16] in *Iris germanica*. The higher growth with that particular combination happened might be due to the selectivity which varied with species to species (Gonbad *et al*., 2014) [5] even explant to explant (Lu *et al*., 1988) [10].

Plant bioregulators have a crucial role in every step of *in vitro* regeneration of Japanese Iris. Data presented in Table 4 found earlier root initiation (26.07 days) *in vitro* in Japanese Iris micro shoots along with greater number of roots per shoot (9.07) having higher length (9.32 cm) were obtained from aseptic rooting medium containing MS + IBA (1.0 mg/l) + NAA (1.0 mg/l). Micro shoots when cultured in MS medium + IBA (1.5 mg/l) + NAA (1.0 mg/l) showed greater percentage of rooted micro shoots (99.62%). Root diameter was found thicker (1.019 cm) with the culture MS medium supplemented with IBA (2.0 mg/l) and NAA (1.0 mg/l). Culture in hormone free MS basal medium showed the least percent (34.96%) of *in vitro* rooting of micro shoots along with most delayed effect. Also, least number of roots per shoot with minimum length and diameter were obtained from culture in hormone free MS basal medium. Similarly, Uzun *et al*. (18) reported successful *in vitro* roots regenerated of micro shoots on MS medium supplemented with IBA and NAA either singly or in combination in *Iris sari* and *I. schachtii*.

Acclimatization of *in vitro* regenerated plantlets is one of the most important steps in plant tissue culture. Amongst the different hardening medium used, equal proportion of vermiculite and vermicompost ($^{v}/_{v}$) as the hardening medium acclimatized the *in vitro* regenerated plantlets earlier (12.45 days) *ex-vitro* with higher survivability percentage (86.85%) whereas, use of equal proportion of garden soil and vermicompost took longer days (19.83) to acclimatize with least survivability (48.15%) (Table 5). Addition of vermicompost in vermiculite enhances the ability of hardening media to supply readily available nutrients to the growing plantlets (Fig. 7). Successful hardening of plantlets using this combination was noticed by Singh *et al*. 2016 [17] in Gerbera; Navnitbhai 2014 [12] in *Psoralea corylifolia* and *Tinospora cordifolia*.

In the conclusion, the present research revealed that the PGRs combination of MS + 2,4-D (1.0 mg/l) + TDZ (0.1 mg/l) showed better plant caulogenesis at minimum days with highest percentage and weight of calli. Similarly, the two other combinations of PGRs i.e., MS + GA_3 (2.0 mg/l) + BAP (1.0 mg/l) and MS + IBA (1.5 mg/l) + NAA (1 mg/l), respectively showed better results for plant organogenesis and rhizogenesis. The ultimate success of the *in vitro* culture plant lies during acclimatization and was found best for media mixture at equal proportion of vermiculite and vermicompost (v/v) with highest survival rate at minimum days.

Table 1: Effect of culture media on callusing intensity and callus morphology of Japanese Iris (*Iris ensata* Thunb.) *in-vitro*

Treatments	Callusing intensity	Callus Morphology
MS	+	Brownish minute
MS+1.0 mg/l 2,4-D	+++	Yellowish nodular
MS+2.0 mg/l 2,4-D	++	Yellowish Friable
MS+3.0 mg/l 2,4-D	++	Whitish Friable
MS+4.0 mg/l 2,4-D	++	Brownish friable
MS+1.0 mg/l 2,4-D + 0.1 mg/l TDZ	+++	Yellowish Friable
MS+1.0 mg/l 2,4-D + 0.2 mg/l TDZ	+++	Greenish Yellow Compact
MS+2.0 mg/l 2,4-D + 0.1 mg/l TDZ	+++	Yellowish Compact
MS+2.0 mg/l 2,4-D + 0.2 mg/l TDZ	+++	Yellowish Compact
MS+3.0 mg/l 2,4-D + 0.1 mg/l TDZ	++	Whitish Friable
MS+3.0 mg/l 2,4-D + 0.2 mg/l TDZ	++	Whitish Friable

MS+4.0 mg/l 2,4-D + 0.1 mg/l TDZ	+	Yellowish Friable
MS+4.0 mg/l 2,4-D + 0.2 mg/l TDZ	+	Yellowish Brown

Table 2: Effect of culture media on callusing characteristics of Japanese Iris (*Iris ensata* Thunb.) *in-vitro*

Treatments	Percentage of callusing explants (%)	Days to callus initiation (days)	Weight of callus at 15 days after initiation (g)	Weight of callus at 30 days after initiation (g)
MS	11.33 (19.60)	119.40	0.023	0.117
MS+1.0 mg/l 2,4-D	93.11 (74.95)	29.57	0.236	1.353
MS+2.0 mg/l 2,4-D	71.33 (57.67)	33.80	0.212	1.222
MS+3.0 mg/l 2,4-D	49.33 (44.62)	40.93	0.216	0.682
MS+4.0 mg/l 2,4-D	34.67 (36.03)	53.27	0.181	0.627
MS+1.0 mg/l 2,4-D + 0.1 mg/l TDZ	96.67 (79.69)	25.30	0.373	2.961
MS+1.0 mg/l 2,4-D + 0.2 mg/l TDZ	91.33 (72.92)	27.40	0.300	2.394
MS+2.0 mg/l 2,4-D + 0.1 mg/l TDZ	84.67 (66.96)	31.73	0.286	1.776
MS+2.0 mg/l 2,4-D + 0.2 mg/l TDZ	82.00 (64.91)	36.17	0.265	1.454
MS+3.0 mg/l 2,4-D + 0.1 mg/l TDZ	52.44 (46.40)	40.60	0.203	0.752
MS+3.0 mg/l 2,4-D + 0.2 mg/l TDZ	46.89 (43.21)	38.67	0.190	0.683
MS+4.0 mg/l 2,4-D + 0.1 mg/l TDZ	37.33 (37.66)	46.83	0.169	0.621
MS+4.0 mg/l 2,4-D + 0.2 mg/l TDZ	32.67 (34.85)	56.23	0.155	0.483
S.E(m)±	0.73	1.52	0.01	0.03
C.D. at 5%	2.93	6.07	0.05	0.12

*Values in the parentheses are the transformed values

Table 3: Effect of culture media on vegetative parameters of Japanese Iris (*Iris ensata* Thunb.) *in-vitro*

Treatments	Days required for callus initiation (days)	Regeneration percent (%)	Number of microshoots per culture	Length of microshoot (cm)	Number of leaves per microshoot	*In-vitro* leaf length (cm)	leaf width (cm)
MS	93.57	17.33 (24.57)	1.60	1.81	3.10	1.48	0.32
MS+0.5 mg/l GA3	50.17	38.00 (38.05)	3.13	2.77	5.00	4.57	0.49
MS+1.0 mg/l GA3	46.93	46.39 (42.96)	2.73	3.13	6.00	6.03	0.85
MS+2.0 mg/l GA3	60.10	43.11 (41.04)	2.37	2.22	4.70	4.68	1.09
MS+0.5 mg/l BAP	40.67	82.22 (65.11)	6.50	4.84	6.67	2.73	1.14
MS+1.0 mg/l BAP	35.00	94.20 (76.09)	7.20	5.88	9.23	3.46	0.89
MS+2.0 mg/l BAP	42.33	87.33 (69.44)	8.30	5.63	11.70	3.81	1.00
MS+2.0 mg/l GA3 + 1.0 mg/l BAP	29.20	99.70 (86.97)	11.60	8.64	7.13	7.00	1.18
MS+2.0 mg/l GA3 + 2.0 mg/l BAP	33.60	99.14 (84.66)	16.30	7.91	9.87	6.43	1.32
MS+2.0 mg/l GA3 + 3.0 mg/l BAP	38.83	92.67 (74.44)	12.60	8.30	8.37	6.27	1.10
MS+3.0 mg/l GA3 + 1.0 mg/l BAP	31.60	75.56 (60.39)	13.47	7.80	7.13	5.38	1.05
MS+3.0 mg/l GA3 + 2.0 mg/l BAP	36.53	55.33 (48.07)	10.30	6.61	8.33	5.21	0.94
MS+3.0 mg/l GA3 + 3.0 mg/l BAP	43.67	46.89 (43.21)	7.40	5.97	7.33	3.75	0.86
S.E(m)±	0.72	2.18	0.22	0.07	0.19	0.32	0.07
C.D. at 5%	2.90	4.81	0.88	0.30	0.78	1.28	0.30

*Values in the parentheses are the transformed values

Table 4: Effect of culture media on rooring parameters of Japanese Iris (*Iris ensata*Thunb.) *in-vitro*

Treatments	Days required for *in-vitro* root initiation (days)	Percentage of micro shoots rooted (%)	Number of roots per micro shoot	Length of root (cm)	Diameter of root (mm)
MS0	64.63	44.96 (42.16)	2.40	2.88	0.298
MS+1.0 mg/l IBA	31.03	94.25 (76.25)	7.60	7.89	0.764
MS+1.5 mg/l IBA	32.40	92.60 (74.16)	6.93	7.49	0.873
MS+2.0 mg/l IBA	35.70	90.06 (71.65)	6.20	6.57	0.929
MS+2.5 mg/l IBA	38.57	98.43 (82.45)	5.37	5.75	0.719
MS+3.0 mg/l IBA	40.47	98.00 (81.92)	4.53	4.93	0.501
MS+3.5 mg/l IBA	41.90	95.00 (77.04)	4.13	3.75	0.478
MS+1.0 mg/l IBA + 1.0 mg/l NAA	26.07	97.22 (80.39)	9.07	9.32	0.920
MS+1.5 mg/l IBA + 1.0 mg/l NAA	29.40	99.62 (86.51)	8.20	8.31	0.980
MS+2.0 mg/l IBA + 1.0 mg/l NAA	33.40	94.01 (75.68)	7.37	7.34	1.019
MS+2.5 mg/l IBA + 1.0 mg/l NAA	34.90	96.71 (79.44)	6.03	6.42	0.836
MS+3.0 mg/l IBA + 1.0 mg/l NAA	36.80	95.98 (78.53)	4.97	4.96	0.527
MS+3.5 mg/l IBA + 1.0 mg/l NAA	40.90	95.75 (78.15)	4.13	3.98	0.516
S.E(m)±	0.59	2.18	0.14	0.18	0.001
C.D. at 5%	2.36	8.71	0.56	0.72	0.016

*Values in the parentheses are the transformed values

Table 5: Effect of different hardening media on plantlets acclimatization of Japanese Iris (*Iris ensata* Thunb.) *ex-vitro*

Treatment code	Treatments	Days required for acclimatization (days)	Hardening Percentage (%)
H1	Vermiculite + sand (1:1)	14.48	85.89 (67.65)
H2	Garden soil + sand+ vermicompost (1:1:1)	17.98	71.45 (57.77)
H3	Vermiculite + vermicompost (1:1)	12.43	86.85 (68.69)
H4	Garden soil + sand (1:1)	15.75	76.75 (61.20)
H5	Soil+ Vermicompost (1:1)	19.83	55.42 (48.15)
	S.E(m)±	0.53	3.19
	C.D. at 5%	2.17	12.76

*Values in the parentheses are transformed values

Fig 1: Callus formation from the leaf base explant

Fig 2: Caulogenesis

Fig 3: Organogenesis

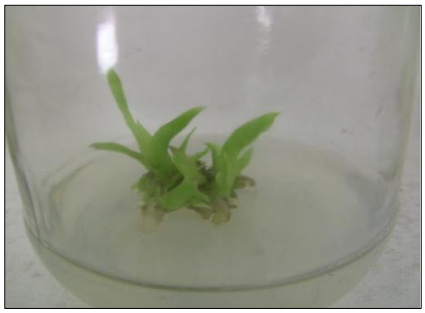

Fig 4: *In vitro* shoot multiplication

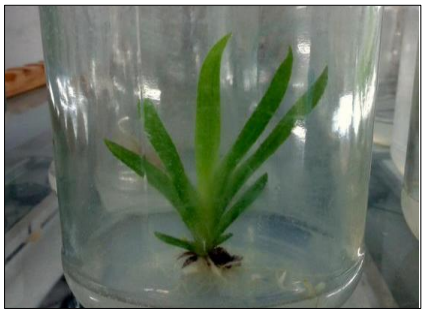

Fig 5: *In vitro* rooted microshoot

Fig 6: Rooted microshoots ready for hardening

Fig 7: Hardened plantlets

Fig 1-7: Different phases of micropropagation of Japanese Iris (*Iris ensata* Thunb.)

Declaration

The authors declare no conflict of interest.

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