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Efficient Micropropagation and Genetic Fidelity Assessment of *In vitro* Raised Dragon Fruit (*Hylocereus* spp.) using ISSR Markers

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Abstract

An efficient *in vitro* regeneration protocol was developed for two dragon fruit species (*Hylocereus polyrhizus* and *Hylocereus undatus*) widely cultivated in Bangladesh. The highest percentage (100%) of explants responded to shoot induction and maximum number of multiple shoots (12.4 ± 1.14 and 14.2 ± 1.14) and highest length of shoots (6.70 ± 1.50 and 5.70 ± 1.50 cm) were obtained in MS + 2.0 mg/l BAP + 0.5 mg/l NAA. The *in vitro* regenerated and proliferated shoots were transferred to ½ strength of MS with 0.8 mg/l NAA where 100% shoots were produced roots. The maximum number (7.5 ± 0.12 and 8.8 ± 0.4) and highest length (7.4 ± 0.3 and 5.8 ± 0.2 cm) of roots were found in this medium. After transplantation to soil the survival rate of *in vitro* regenerated plantlets was 100%. No ISSR polymorphism in the micropropagated plants was detected, while 3 Inter Simple Sequence Repeat (ISSR) markers were used in order to look for any somaclonal variation among *in vitro*-derived plants, demonstrating the high level of genetic fidelity in the plantlets produced by the regeneration technique reported in this work.

Introduction

Dragon fruit (*Hylocereus* spp.) is an important table fruit valued for its attractive appearance, vibrant color and high nutritional content. Owing to its richness in bioactive compounds, antioxidants, vitamins and dietary fiber, dragon fruit has gained increasing importance not only as a fresh fruit but also as a valuable raw material for the food and pharmaceutical industries (Jimenez-Garcia et al. 2022). Dragon fruits originate from southern Mexico, the Pacific side of Guatemala and Costa Rica, and El Salvador (Francis Zee 2004). Indonesia, China and Vietnam are produced 93% dragon fruit worldwide

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(Sharma et al. 2021). Dragon fruit belongs to the family of Cactaceae and has a few species. Among them *Hylocereus undatus* (red skin with white flesh), *Hylocereus polyrhizus* (red skin with red-violet flesh), *Hylocereus costaricensis* (red skin with red flesh), *Selenicereus megalanthus* (yellow skin with white flesh) are cultivated worldwide (Nerd et al. 2002). It is also known as pitaya, pitahaya, strawberry pear, and night-blooming cereus around the world. Dragon fruits are used to make jams, drinks, ice cream etc. and it contains vitamins, dietary fiber, glucose, and fructose (Fan et al. 2013). *Hylocereus spp.* also have medicinal values including hypoglycemic, diuretic, and cicatrizing aspect (Kakade et al. 2020). In Bangladesh, dragon fruit was initially introduced by private entrepreneurs from various countries, primarily for ornamental cultivation. Subsequently, commercial cultivation was initiated by elite farmers in different regions of the country, including Ashulia (Dhaka) and the Halda Valley Tea Estate in Fatikchari, Chattogram (Patwary et al. 2013). Conventional propagation methods such as vegetative cuttings and seed propagation are widely practiced. However, these methods are constrained by limited multiplication rates, extended juvenile periods, and genetic heterogeneity among progeny (Fan et al. 2013). Establishing efficient *in vitro* propagation protocols is crucial for rapid multiplication of genetically uniform and disease-free planting materials. Micropropagation offers a reliable alternative to conventional systems by enabling rapid production of uniform plants under controlled, sterile conditions. Techniques such as temporary immersion bioreactors have been shown to greatly enhance the efficiency of i*n vitro* propagation by improving nutrient uptake and proliferation rates compared with traditional semisolid media (Bello-Bello et al. 2021). Advances in shoot regeneration protocols have also demonstrated high regeneration rates from explants of dragon fruit cultivars, indicating significant potential for commercial application (Carmona-Martín et al. 2025). Although several *in vitro* regeneration protocols for *Hylocereus* species have been reported (Drew and Azimi 2002, Mohamed-Yasseen 2002), regeneration efficiency often varies among cultivars due to genotype-specific responses to culture conditions. Studies on dragon fruit have shown that optimized hormone combinations and explant types are necessary for efficient shoot proliferation and rooting (Fan et al. 2013). Furthermore, protocols often need to be adapted when new cultivars are introduced to different agro-ecological regions (Ismail et al. 2023). For this, the present study aimed to establish a simple, rapid, and high-frequency *in vitro* regeneration protocol using shoot tip explants to produce true-to-type clones for large-scale propagation for introduced cultivars in Bangladesh. Genetic fidelity of the regenerated plants was also assessed using ISSR molecular markers to confirm clonal uniformity.

Materials and Methods

Red dragon (*Hylocereus polyrhizus)* fruits and white dragon (*Hylocereus undatus*) fruits were collected from local market of Dhaka. The fruits were washed with detergent for half an hour under running tap water. The samples were then washed with Tween 80

and 2% carbendazim for 25 min and 20 min respectively. 0.1% $HgCl_2$ was used as surface sterilization for 15 min in laminar airflow. Autoclaved distilled water was applied for several times in every steps of sterilization. The fruits were cut properly and seeds were separated and collected. Seeds were then transferred to sterile water soaked cotton bed for germination. After germination shoot tips were excised from the newly developed shoots and were used as explant. For *in vitro* regeneration explants were transferred to MS medium supplemented with different concentrations and combinations of BAP, Kn and NAA. After the initiation of regeneration, the proliferated micro-shoots were subcultured at every 4 weeks of interval. All the cultures were kept in growth room at 25 ± 2°C temperature, 28 μmol/m^2/s (White LED) and 16/8 hrs photoperiod. For induction of roots, the regenerated shoots were excised and transferred to half-strength of MS with different concentrations of IBA, IAA and NAA. After the formation of roots, the plantlets were transferred to small plastic pots containing 1 : 1 (v/v) mixture of soil and farmyard manure and covered with perforated polythene bags for acclimatization. After three weeks the acclimated plant were transferred to experimental field for further growth and development. A total of 10 randomly *in vitro*-grown plants of two genotypes and a mother plant for both species were used to evaluate genetic stability using Inter Specific Sequence Repeats (ISSR) (Fan et al. 2013). Shoots from regenerated plantlets were collected and DNA were isolated with CTAB protocol (Li et al. 2013) and kept at -20°C. ISSR amplification reactions were carried out with 45 cycles comprising 50 ng DNA templates. The denaturation temperature ranged from 37-48°C depending on the primer sequence (Table 1). The PCR amplified DNA fragments were separated using 0.8% agarose gel electrophoresis and documented by gel documentation system.

Table 1. Primer configurations and annealing temperatures in *in vitro* regenerated dragon fruit shoots utilized for ISSR detection.

Primer code	Sequence (5'-3')	Tm (°C)
UBC-835	$(AG)_8$YC	48
UBC-845	$(CT)_8$RG	48
UM14-T4	$(CA)_6$GT	37

Y= C or T, R= A or G.

Results and Discussion

In this study, shoot tip explants from *in vitro* germinated seedlings were used to test the possibility of direct shoot regeneration of dragon fruit. Fresh seed showed higher viability in *in vitro* condition than stored seeds of three months old. The germination rate of *H. undatus* (white dragon) and *H. polyrhizus* (red dragon) were almost same. Shoot tip explants were cultured on MS with different concentrations of BAP (1.0-2.5 mg/l) and Kn (1.0-2.5 mg/l) singly and combinations with different concentrations of NAA (0.5-1.0 mg/l) for *in vitro* shoot regeneration. Both cytokinins significantly affected the regeneration of adventitious buds from the shoot tip explants as compared to the cytokinin free controls.

Table 2. Effects of various concentrations and combinations of BAP, Kn and NAA on *in vitro* regeneration and proliferation of *H. undatus* (white dragon) and *H. polyrhizus* (red dragon) from shoot tip explant (data were recorded after six weeks of culture).

Growth regulators (mg/l)			Species	% of responsive explants	Days for initiation of regeneration	No. of shoots/explant Mean ± SE	Length of shoots (cm.) Mean ± SE
BAP	Kn	NAA					
1.0	-	-	WD*	80	13	5.1 ± 1.3	3.70 ± 0.35
			RD*	80	12	6.1 ± 1.7	3.20 ± 0.65
1.5	-	-	WD	88	11	5.2 ± 1.4	4.70 ± 0.65
			RD	98	10	6.2 ± 1.4	3.70 ± 0.45
2.0	-	-	WD	100	10	7.3 ± 0.94	5.40 ± 0.90
			RD	100	8	8.3 ± 0.94	5.30 ± 0.90
2.5	-	-	WD	90	7	6.2 ± 0.14	4.90 ± 0.75
			RD	95	5	7.2 ± 1.34	3.90 ± 0.75
	1.0	-	WD	70	14	1.8 ± 0.70	4.20 ± 1.65
			RD	70	13	1.8 ± 0.70	4.40 ± 1.65
	1.5	-	WD	80	12	3.2 ± 1.4	3.90 ± 0.45
			RD	75	12	3.1 ± 1.4	3.90 ± 0.45
	2.0	-	WD	95	11	5.3 ± 0.94	5.50 ± 0.9
			RD	85	10	5.0 ± 0.94	5.20 ± 0.9
	2.5	-	WD	90	8	5.8 ± 0.34	4.20 ± 0.55
			RD	80	8	2.7 ± 0.34	4.50 ± 0.55
1.0	0.5	-	WD	35	13	3.8 ± 1.18	3.10 ± 1.44
			RD	35	12	3.8 ± 0.18	3.10 ± 1.44
2.0	0.5	-	WD	80	12	7.1 ± 1.34	6.30 ± 1.19
			RD	80	11	7.3 ± 1.04	4.30 ± 1.90
2.0	1.0	-	WD	76	13	4.7 ± 0.98	5.60 ± 0.50
			RD	76	10	6.2 ± 0.88	3.60 ± 0.95
1.0		0.5	WD	90	8	5.0 ± 0.68	5.60 ± 0.74
			RD	90	10	5.2 ± 0.68	4.50 ± 0.74
2.0		**0.5**	**WD**	**100**	**9**	**12.4 ± 1.14**	**4.70 ± 1.50**
			RD	**100**	**7**	**14.2 ± 1.14**	**5.70 ± 1.50**
2.0		1.0	WD	85	12	3.7 ± 1.24	5.70 ± 1.75
			RD	100	5	6.5 ± 0.37	5.20 ± 0.65

*WS = white dragon, RD= red dragon.

Initial shoot induction and subsequent shoot growth were observed on MS medium supplemented with 2.0 mg/l BAP. At this concentration, shoot initiation began within 8-10 days, and the highest response (100%) of explants was recorded for shoot induction in both red- and white-fleshed dragon fruit species. The number (7.3 ± 0.94 and 8.3 ± 0.94) of shoots/explant and length (5.40 ± 0.90 and 5.30 ± 0.90 cm) of shoots were recorded in this concentration in case of both species of dragon fruit, respectively (Table 2). Similar findings were also reported by Fan et al. (2013) in plant regeneration and bud

proliferation of dragon fruit. In the present investigation, BAP showed better response to multiple bud formation as compared to Kn. Sashikumar et al. (2009) also reported that among the different cytokines, the highest rate of adventitious bud regeneration was found in BAP in the case of dragon fruits. The initiation of regeneration was obtained within 10 to 11 days in MS supplemented with 2.0 mg/l Kn. A response rate of 95% in white dragon fruit and 85% in red dragon fruit explants was observed on MS medium supplemented with 2.0 mg/l Kn. The number of shoots per explant (5.3 ± 0.94 and 5.0 ± 0.94), along with the average shoot length (5.50 ± 0.90 and 5.20 ± 0.90 cm), was obtained on MS medium supplemented with 2.0 mg/l Kn in the two dragon fruit species, respectively.

The combined effect of BAP, Kn and NAA in MS was observed for their synergistic effect on direct shoot induction from shoot tip explants of both species of dragon fruit. When shoot tips were cultured on MS supplemented with 2.0 mg/l BAP and 0.5 mg/l Kn a considerable number of explants responded to regeneration in case of both species of dragon fruits. Shoot regeneration was initiated within one week after of explants inoculation (Table 2).

MS medium supplanted with BAP and NAA at different concentrations was also used to observe the shoot induction in the explants of both species of dragon plants (Table 2). Higher concentration of BAP combined with lower NAA concentration increased the regeneration frequency in dragon fruit. The highest percentage (100%) of responded explant was observed in MS medium containing 2.0 mg/l BAP and 0.5 mg/l NAA. Among different regeneration media MS with BAP and NAA showed the best regeneration efficiency in case of both species of dragon fruit. The explants of both species of dragon grew rapidly and vigorously in MS supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA. The initiation of regeneration occurred after six days of explant inoculation. The maximum number (12.4 ± 1.14 and 14.2 ± 1.14) of shoots per explant and shoot length (6.70 ± 1.50 and 5.70 ± 1.50 cm) were found in this combination in case of both species of dragon respectively. The developing cultures were subcultured at a regular interval of three to four weeks. After four to five subcultures more than 300 microshoots were observed in a single culture vessel. Aygun and Dumanoglu (2015) found the highest percentage of shoot regeneration on media supplemented with 2.0 μM BAP combined with 0.5 μM IAA.

For root induction, shoots were excised from the shoot clusters individually and transferred to different strength of MS or in combinations with various concentrations of NAA, IBA and IAA (0.4-0.8 mg/l) singly. The kind or amount of auxins were accelerated the root induction in *in vitro*-derived shoots as compared to the auxin-free half and full strength of MS medium (Table 3). Among the different auxins (IBA, IAA and NAA) the best root initiation and subsequent growth of roots was found in half strength of MS medium containing 0.8 mg/l NAA. The highest percentage (100%) of root formation, maximum number of roots (7.5 ± 0.12 and 8.8 ± 0.4) and highest length (7.4 ± 0.3 and 5.8 ± 0.2 cm) of roots were recorded in this medium within three weeks of inoculation in case

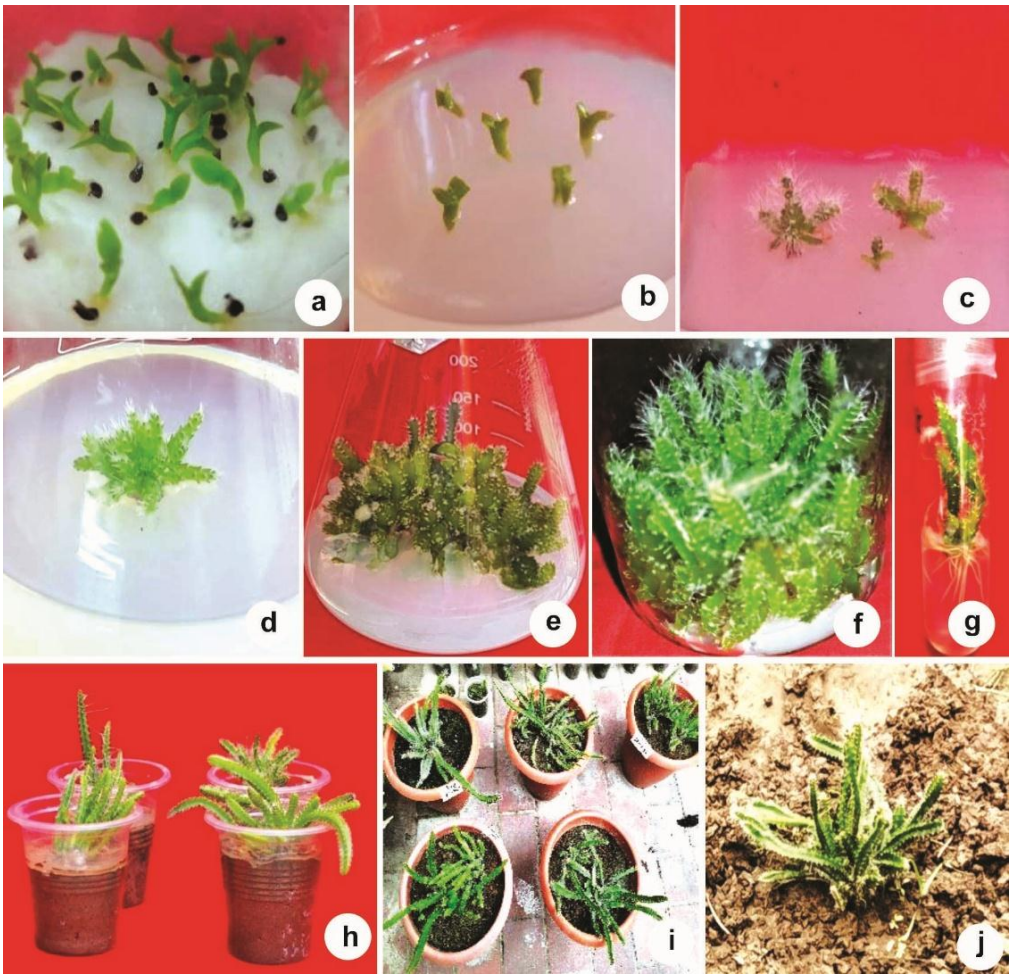

Fig. 1(a-j) *In vitro* multiple shoot regeneration of two species of Dragon from shoot tip explants: (a) *In vitro* germination of seedlings in water soaked cotton bed to, (b) shoot tip explants were transferred regeneration medium, (c) initiation of shoot buds from shoot tip explants of white dragon fruit on MS with 2.0 mg/l BAP + 0.5 mg/l NAA, (d) proliferation of *in vitro* regenerated shoots of red dragon fruit after sub-culture in MS with 2.0 mg/l BAP + 0.5 mg/l NAA medium, (e) multiplication shoots on same regeneration medium after sub sequent subculture of white dragon , (f) vigorous growth of well-developed shoots after 2 months of culture initiation in on MS with 2.0 mg/l BAP + 0.5 mg/l NAA in case of red dragon fruit, (g) fully developed roots at the base of regenerated shoots on ½MS medium with 0.8 mg/l NAA in red dragon, (h & i) acclimatization of *in vitro* grown plantlet in a small and large plastic pot containing soil of white dragon fruit, and (j) *In vitro* derived plant of red dragon fruit transferred to experimental field for evaluation and further growth.

of both white and red dragon fruit respectively (Fig. 1). Yasseen (2002) also reported half strength of MS media solidified with agar for induction of roots from *in vitro*-derived shoots of dragon fruit. After sufficient development of roots, the plantlets obtained from two dragon fruit species were transferred to small plastic pots containing garden soil and

farmyard manure (1 : 1) for hardening. In contrast, Kabir et al. (2024) used a mixture of soil and compost (2 : 1) for hardening. The survival rate of the transplanted plantlets was found to be about 100%. After three weeks of transplantation the plantlets were transferred to large plastic pots and kept in net house. In net house plants started to elongate and produce new branches. Then the well developed elongated plantlets of both species of dragon fruit were transplanted to experimental field for their further growth and development.

Table 3. Effects of IBA, IAA and NAA on *in vitro* root induction in regenerated shoots of dragon fruit on half and full-strength MS (data were recorded after four weeks of culture).

MS strength	Growth regulators (mg/l)			Species	Rooted shoots (%)	Days required for initiation of rooting	No. of roots/ Shoot	Length of roots (cm)
	IBA	IAA	NAA				Mean ± SE	Mean ± SE
½MS	-	-		WD*	0	0	0	0
				RD*	0	0	0	0
½MS	0.4	-		WD	90	12	5.7 ± 0.30	5.8 ± 0.20
				RD	94	11	6.7 ± 0.35	4.8 ± 0.20
½MS	**0.8**	-		**WD**	**100**	**11**	**6.5 ± 0.13**	**6.2 ± 0.30**
				RD	**100**	**9**	**7.5 ± 0.43**	**5.1 ± 0.10**
½MS	-	0.4		WD	85	16	5.5 ± 0.30	5.7 ± 0.10
				RD	90	15	5.8 ± 0.33	4.4 ± 0.20
½MS	-	0.8		WD	80	13	6.8 ± 0.22	6.4 ± 0.40
				RD	94	14	6.8 ± 0.20	5.1 ± 0.30
½MS			0.4	WD	96	12	6.2 ± 0.20	6.5 ± 0.32
				RD	98	10	7.6 ± 0.30	5.3 ± 0.38
½MS			0.8	WD	100	11	7.5 ± 0.12	7.4 ± 0.30
				RD	100	8	8.8 ± 0.40	5.8 ± 0.20
MS	-	-		WD	0	0	0	0
				RD	0	0	0	0
MS	0.4	-		WD	80	16	6.2 ± 0.33	4.8 ± 0.20
				RD	94	15	6.2 ± 0.33	3.8 ± 0.20
MS	0.8	-		WD	90	15	6.5 ± 0.20	5.2 ± 0.10
				RD	95	14	6.5 ± 0.20	4.7 ± 0.10
MS	-	0.4		WD	60	22	5.2 ± 0.23	5.2 ± 0.13
				RD	80	20	5.8 ± 0.30	4.2 ± 0.10
MS	-	0.8		WD	70	21	6.2 ± 0.20	5.8 ± 0.30
				RD	90	18	6.0 ± 0.40	5.0 ± 0.20
MS			0.4	WD	90	17	5.2 ± 0.34	5.0 ± 0.40
				RD	92	14	6.2 ± 0.20	5.0 ± 0.40
MS			0.8	WD	93	15	6.0 ± 0.21	5.1 ± 0.20
				RD	94	14	7.1 ± 0.20	5.1 ± 0.20

*WS = white dragon, RD= red dragon.

Differences in regeneration responses reported among studies may be attributed to several biological and experimental factors. Genotypic variation among species or cultivars of *Hylocereus* can strongly influence their response to plant growth regulators and *in vitro* culture conditions, resulting in variations in shoot proliferation efficiency (Fan et al. 2013). Furthermore, type and physiological condition of explants and differences in culture conditions, including nutrient composition of the medium and plant growth regulator combinations, may also contribute to variations in regeneration efficiency observed in different studies (George et al. 2008).

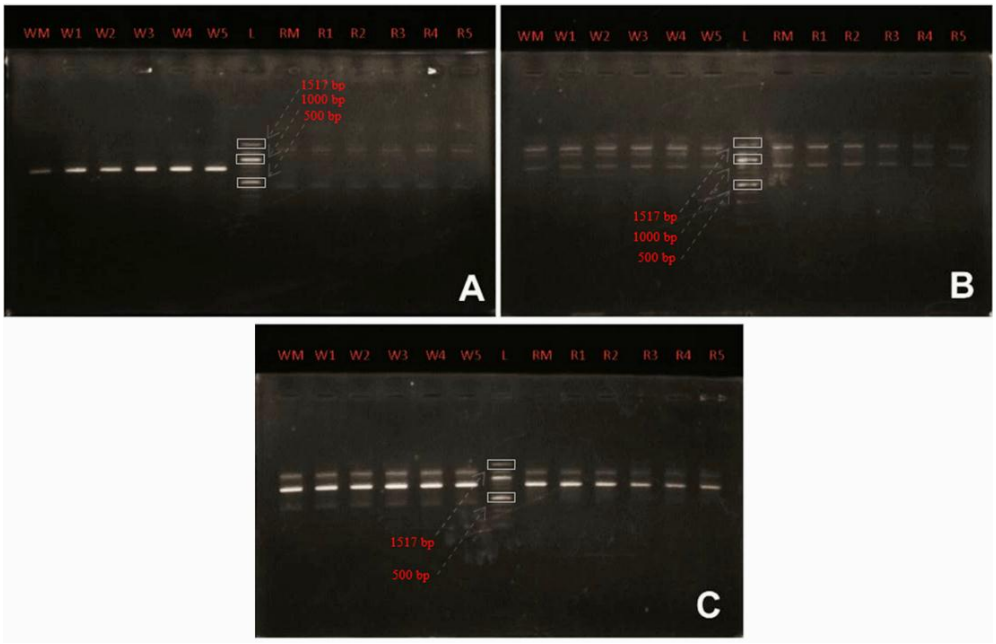

Fig. 2(A-C). Representative picture of polymerase chain reaction (PCR) amplification obtained with inter simple sequence repeat (ISSR) primers from *in vitro* propagated plants and their mother. Primers used: (A) ISSR-UBC 845, (B) ISSR-UM14T4, and (C) ISSR-UBC835. Lane L represents 100 bp ladder, lane WM and RM represents the white and red dragon mother plant, lanes W1-W5 and R1-R5 represent *in vitro*-raised clones derived from shoot tip explant of white and red dragon fruits, respectively.

Genetic diversity analysis using ISSR markers was carried out among the micropropagated plants derived from the *in vitro* culture of the dragon plants. It is generally known that molecular markers can be used to evaluate the genetic fidelity of plants that have been grown *in vitro* (Wen and Deng 2005, Khan et al. 2009, Miguel and Marum 2011, Tripathi et al. 2012). To ensure the genetic stability of the micropropagated plants 3 ISSR primers were used to identify the genetic variability among the 5 phenotypically similar micropropagated plants of each species and field grown mother plants of the both species. A total of 13 scorable bands were generated by the three ISSR primers, with band sizes ranging approximately from 400 bp (UBC 845) to 1500 bp

(UM14T4). All the 3 ISSR primers produced uniform bands confirming the genetic homogeneity of the *in vitro*-raised plants (Fig. 2). Bairu et al. (2011) also reported of genetically identical micropropagated plant of dragon fruit. The observed genetic homogeneity among the *in vitro* regenerated plants indicates that the micropropagation protocol preserved clonal fidelity. In micropropagation systems, genetic uniformity is expected when regeneration occurs through organized meristematic tissues (e.g., shoot tips) which minimizes the risk of somaclonal variation. The ISSR profiles obtained in this study showed monomorphic banding patterns identical to the mother plant, confirming the absence of detectable genetic variation. Similar findings have been reported in other species, where ISSR and RAPD markers revealed monomorphic bands and high similarity among regenerants and donor plants, indicating genetic stability (Bisht et al. 2024). Molecular marker analysis is widely considered a reliable approach for validating clonal fidelity, as it can detect even minor genetic changes induced during *in vitro* culture. Therefore, the absence of polymorphism in the present study confirms that the developed protocol is suitable for producing true-to-type planting materials of *H. polyrhizus* and *H. undatus* to serve as a convenient and useful technique for the mass propagation of this highly nutritional fruit plant.

An efficient and reproducible *in vitro* regeneration protocol was successfully established for two commercially important species of dragon fruit, *Hylocereus undatus* and *Hylocereus polyrhizus*. MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA proved to be the best for shoot induction and multiplication, while ½ strength of MS with rates of 0.8 mg/l NAA facilitated successful rooting. The regenerated plantlets showed high survival rates during acclimatization and plantlets were successfully established in soil under net house and field conditions. This protocol provides a reliable platform for large-scale clonal propagation, germplasm conservation, and future genetic improvement of dragon fruit in Bangladesh.

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