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Efficient *in vitro* Propagation Protocol for Mass Multiplication in Dragon Fruit (*Hylocereus costaricensis*)

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ABSTRACT: Dragon fruit, *Hylocereus costaricensis* (Cactaceae) was once widely grown as an ornamental plant but it is now considered as emerging fruit crop due to its neutraceutical values and consumer preference. It is commercially propagated by cuttings. The present study was carried out to find out an efficient and viable *in vitro* propagation protocol for mass multiplication of dragon fruit through direct organogenesis. The shoot tips of different length (1.5, 2.0, 2.5 cm) were cultured on Murashige and Skoog (MS) media with different concentrations of growth regulators such as BAP and cytokinin under *in vitro* conditions. Results revealed that highest *in vitro* response was recorded with shoot tips of 2cm in length supplemented with 2mgL^{-1} of BAP. Highest number of multiple shoots was reported in MS media supplemented with 7mgL^{-1} of BAP and 1mgL^{-1} of Kinetin. Further, maximum shoot length (3.5cm) was recorded in MS media supplemented with 7mgL $^{-1}$ of BAP and 1mgL $^{-1}$ of kinetin compared to others. Hence from the present study, it is inferred that MS medium fortified with 7mg L^{-1} BAP and 1 mgL^{-1} Kinetin were found to be the best combination in terms of time taken for multiple shoot induction, number of multiple shoots and shoot length. The present study is focused on overcoming the challenges of *in vitro* propagation and raising genetically homogeneous and true to type plantlets in order to address the main problems encountered by researchers in plant tissue culture, which are mainly contamination and phenolic browning. Therefore, it is anticipated that this protocol is effective and that it can be utilized to generate dragon fruit in large quantities for commercial purposes.

Keywords: *Hylocereus costaricensis*, Dragon fruit, Mass multiplication, *In vitro* micropropagation.

INTRODUCTION

Dragon fruit (*Hylocereus costaricensis* L.) is a cactus, belonging to the family Cactaceae. Recently, dragon fruit is introduced as super fruit in India and it is considered to be a promising and remunerative fruit crop. It is a long day plant with beautiful night blooming flower that is nicknamed as "Noble Woman" or "Queen of the Night". The fruit is also known as straw berry pear, dragon fruit, pithaya, night blooming cereus, Belle of the night, Conderella plant and Jesus in the Cradle. Fruit is named as pitaya because of the bracts or scales on the fruit skin and hence, the name of pitaya meaning 'the scaly fruit'. It has ornamental value due to the beauty of their large flowers (25 cm) that bloom at night; they are creamy white in colour. Dragon fruit production is gaining importance and it is receiving more recognition as a commercial fruit crop in India. It is commercially propagated by cuttings and however multiplication rates are very low and obtaining enough planting material is difficult due to the large size of the cuttings required (50 cm lengths) (Le Bellec *et al*., 2006). Despite seed germination efficiencies of 71 to 83 percent for *H. undatus* (El Obeidy, 2006), such propagation is not commercially viable because seed-derived plants have a long juvenile period and delaying fruit production for several years.

However, through tissue culture, production of a large number of clonal plants in a relatively short period of time is possible while using very small plant material (Rubluo *et al*., 1993). *Hylocereus undatus* can develop branching in its younger sections, while its basal parts can only produce callus. Explants should be chosen according to their age and place of origin, whether they are from in vivo mature plants or in vitro germinated seedlings (Garcia-Rubio and Malda-Barrera 2010). The selection of appropriate explants and medium is the most important consideration for enhanced dragon fruit *in vitro* response. Thinesh and Seran (2015) used dragon fruit bud and stem explants, Wyka and Ludwiczak (2009) used flower buds from *Mammillaria* sp., and Caetano Nunez *et al*. (2014) reported using *Selenicereus megalanthus* explants from meristematic tissue. Exudation of phenolic compounds from plant tissue cut ends causes browning of explants and has proven fatal to culture establishment. Browning in plants is caused primarily by phenol oxidase results in oxidation of phenolic compounds. Application of antioxidants in the medium and selection of suitable explants have been tested in order to overcome

browning by many workers in Strawberry (Hidayatullah Mir *et al*., 2019); Guava (Ahmad *et al*., 2016); Banana (Ko *et al*., 2009).

Many studies have been conducted by using *in vitro*-germinated plants to avoid disinfection of explants due to extreme sensitivity to common disinfection procedures and subsequent low survival rates (Santos-Diaz *et al*., 2003). Extensive research on the composition of mineral salts, plant growth regulator and chemical compounds in culture media is usually required when developing effective micropropagation processes for a specific species.

The focus of this study was to develop an efficient and viable protocol for *in vitro* establishment and vegetative propagation of *Hylocereus costaricensis*, a betalains-rich pink fleshed pithaya (Esquivel *et al.*, 2007), using different-sized shoot tips as explants. Furthermore, it describes a successful regeneration protocol for the formation of multiple shoots from *in vitro* derived plants.

MATERIALS AND METHODS

The present study was carried out at Tissue culture laboratory, Department of Fruit Science, Horticultural College and Research Institute, TNAU, Coimbatore during 2021-2022 to develop an efficient method for mass multiplication of dragon fruit plants under *in vitro* conditions through direct organogenesis.

Planting material. Young shoot tips (1.0, 1.5, 2.0, 2.5 cm in length) with 4 to 5 nodes of dark green colour shoots were collected from two years old healthy mother plants of dragon fruit plants maintained at University orchard, Horticultural College and Research Institute, TNAU, Coimbatore as explants for *in vitro* propagation of dragon fruit.

Sterilization of explants. The shoot tip explants were collected from the field and then washed thoroughly under running tap water and then pretreated with tween 20 for 5 minutes and then with fungicide treatment such as Bavistin 0.1% for 15 minutes and then subsequently washed with tap water and distilled water. After that under laminar air flow chamber the explants were given a brief wash with 70 percent ethanol, followed by three sterile water washes and then with 0.1% mercuric chloride for 3 to 5 minutes followed by 3 to 4 sterile water wash.

Culture medium. The surface sterilized explants were inoculated on Murashige and Skoog mineral salts (MS) medium supplemented with thiamine hydrochloride (0.1 mgL^{-1}), pyridoxine hydrochloride (0.5mgL^{-1}), nicotinic acid (0.5mgL^{-1}), glycine (2 mgL^{-1}), myo-inositol (100 mgL^{-1}), sucrose (30gL^{-1}), plant growth regulators such as 6-Benzyl Amino purine (BAP), Kinetin and antioxidants like citric acid (100mgL^{-1}) and ascorbic acid (100mgL^{-1}). The pH was adjusted to 5.6 to 5.8 with KOH/ NaOH, and the medium was gelled with (0.8 %) agar poured into 25× 150 mm culture tubes and culture bottles and the media was sterilized by autoclaving under 121°C for 15 minutes at 15 psi.

Inoculation of explants. Tip section of the sterilised shoot tips (1.5, 2.0 and 2.5 cm in length) was removed by using scalpel and forceps under laminar air flow chamber. Following that, the excised explants were placed on MS media containing the plant growth regulators described above as well as 30 gL^{-1} sucrose. Each culture vial containing four explants were labelled and parafilm strips were used to seal it.

Culture Environment. The cultured bottles containing explants were incubated at temperature of 25±2°C, 60 to 70% relative humidity, with a photoperiod of 16 hours of light and 8 hours of darkness with 3000 lux light intensity using white fluorescent light. The cultures were checked daily for contamination and subsequently photoperiod and temperature were maintained inside the culture room.

Shoot initiation. Aseptic culture initiation was the initial procedure for *in vitro* establishment. The shoots formed in MS basal medium with various concentrations of growth hormones (1, 1.5, 2.0, 2.5, and 3 mgL^{-1} of BAP). Shoots were also formed in MS basal medium without growth hormones served as control. However, maximum shoot initiation takes place in MS media supplemented with 2 mgL^{-1} BAP (T_3) compared to other treatments (Table 1). Shoot regeneration usually takes 23 to 35 days after inoculation into the culture medium be cause of its slow growing and succulent in nature. During the incubation phase, browning of explants were observed in few culture bottles. Subculture was done once in two weeks to keep away the explants from browning.

Mass Multiplication. Following establishment on the initiation media, the explants were transferred into multiplication media for further proliferation. Shoots of 1.5cm in length was achieved after 35 days of inoculation (Table 3) and it was transferred into a medium containing different concentrations of cytokinins such as Benzyl Amino purine (BAP) (3mgL^{-1} to 7mgL^{-1}) and kinetin (1mgL^{-1}) to get multiple shoot formation. The MS media supplemented with 7 mgL^{-1} BAP +1 mgL^{-1} Kinetin (Table 4) and 30 gL^{-1} sucrose, produced maximum number (7) of multiple shoots and maximum length (3.5cm) of shoots were recorded after 25 days of subculturing and it is repeated for every 20 days. Multiple shoots with identical numbers were observed in BAP concentrations of 3 and 4 $mgL^{-1}$and 1mgL^{-1} Kinetin (Table 4).

Table 1: Treatments with different plant growth regulator combinations used for shoot proliferation.

Treatments	MS media with growth regulator combinations(mgL^{-1})
T_0	MS Basal
T_1	MS + 1 mgL^{-1}BAP
T_2	MS+ 1.5 mgL^{-1} BAP
T_3	MS + 2 mgL^{-1} BAP
T_4	MS + 2.5 mgL^{-1} BAP
T_5	MS + 3 mgL^{-1}BAP

Table 2: Effect of treatments on sterilization of explants.

Treatments	Surfacesterilization concentrations (%)	Survival (%)	Contamination (%)	Mortality (%)
T_1	0.1% Bavistin + $HgCl_2$ (0.1% for 1 min)	60 (0.64)	80 (0.92)	45 (0.46)
T_2	0.1% Bavistin + $HgCl_2$ (0.1% for 2 min)	65 (0.70)	70 (0.77)	42 (0.43)
T_3	0.1% Bavistin + $HgCl_2$ (0.1% for 3 min)	70 (0.77)	55 (0.58)	40 (0.41)
T_4	0.1% Bavistin) + $HgCl_2$ (0.1% for 4 min)	50 (0.52)	65 (0.70)	47 (0.48)
T_5	0.1% Bavistin + $HgCl_2$ (0.1% for 5 min)	40 (0.41)	75 (0.84)	49 (0.51)
T_6	0.1% Bavistin + $HgCl_2$ (0.1% for 6 min)	30 (0.30)	60 (0.64)	51 (0.53)
SED		**0.02**	**0.03**	**0.02**
CD (0.05)		**0.06**	**0.09**	**0.05**
CV(%)		**6.78**	**6.53**	**6.55**

Table 3: Effect of Cytokinins on *in vitro* shoot proliferation using shoot tips as explants.

Treatment	Time taken for shoot initiation (days)
MS + BAP ($1mgL^{-1}$)	40
MS + BAP ($1.5mgL^{-1}$)	38
MS + BAP ($2mgL^{-1}$)	23
MS + BAP ($2.5mgL^{-1}$)	32
MS + BAP ($3mgL^{-1}$)	35
Control (MS basal)	45
SED	**1.40**
CD (0.05)	**4.33**
CV (%)	**6.87**

Table 4: Effect of Plant growth regulators on *in vitro* shoot multiplication using shoot tips as explants.

Treatment	Time taken for multiple shoot induction (days)	Number of multiple shoots/shoot	Shoot length (cm)
MS + BAP ($3mgL^{-1}$) + $1mgL^{-1}$ Kinetin	67	1	2.7
MS + BAP ($4mgL^{-1}$) + $1mgL^{-1}$ Kinetin	60	2	2.8
MS + BAP ($5mgL^{-1}$) + $1mgL^{-1}$ Kinetin	55	3	3
MS + BAP ($6mgL^{-1}$) + $1mgL^{-1}$ Kinetin	50	4	3.2
MS + BAP ($7mgL^{-1}$) + $1mgL^{-1}$ Kinetin	45	7	3.5
SED	**2.11**	**0.144**	**0.11**
CD (0.05)	**6.67**	**0.45**	**0.36**
CV (%)	**6.62**	**7.34**	**6.65**

Statistical Analysis. This experiment was carried out in Completely randomized design (CRD) with three replications in each treatment and observations recorded as percentage were subjected to arc sine transformation. Data was recorded at regular intervals and was analysed using ANOVA at 5% level of significance and MS Excels preadsheet.

RESULTS AND DISCUSSION

Selection of suitable explants is an important feature of commercial tissue culture which will yield true to type plants and it may influence the production of number of shoots per explant. Success of *in vitro* propagation through direct organogenesis depends on type of explants and concentrations of plant growth regulators used. Different types of explants were reported such as bud and stem explants (Thinesh and Seran 2015) Young joints (20 to 50mm) in length (Mohamed-Yasseen, 2002). Karimi *et al*. (2010) studied the callus induction of *Cereus peruvianus* using stem explants. Ghaffari *et al*., (2013) reported that young cladodes of *Opuntia* sp were used as explants and can successfully regenerate shoots. In the present study, the shoot tips of different sized segments of dragon fruit were used as explants and it was cultured on MS media supplemented with five different concentrations (1.0, 1.5, 2.0, 2.5 and 3 mgL^{-1}) of BAP to determine the most suitable concentrations for shoot initiation (Table 1).

Kasim *et al*. (2019) developed a protocol for multiple shoot regeneration using stem explants of *Hylocereus costaricensis* cultured on Murashige and Skoog medium supplemented with $3mgL^{-1}$ of BAP and

0.5mgL^{-1} NAA and exhibited highest *in vitro* response for callus formation and highest number of shoots were produced. The present study results concluded that the positive response of shoot initiation took place when shoot tips of 2.5cm were cultured on MS media supplemented with 2mgL^{-1} BAP. Survival percentage were over 70 % in T_3 (O.1% Bavistin + $HgCl_2$ 0.1% for 3 min) and it recorded the highest survival percentage compared to other treatments (Table 2). Less contamination was recorded in T_6 (0.1% Bavistin + $Hgcl_2$ (0.1% for 6 min) and it was highest (80%) in T_1 (0.1% Bavistin + $HgCl_2$ (0.1% for 1 min) (Table 2). Further, it was noted that the survival percentage of shoot tip explants was 65% at the second week afterwards, it reduced to 58% at the third week. Shoot tips of 1.5 cm taken as explants exhibited lower contamination than 2.0cmand 2.5cm. Lowest mortality percentage (40%) was recorded in T_3 (0.1% Bavistin + $HgCl_2$ (0.1% for 3 min) and it was highest (51%) in T_6 (0.1% Bavistin + $Hgcl_2$ (0.1% for 6 min) (Table 2).

The microbes present in plant tissues commonly resulted in decreasing survival rate of the cultured explants, *in vitro* morphogenic response and their variable growth (Kane 2003). Browning of explants were also observed in a few cultures during their incubation. It is generally due to phenolic compounds that are released from the cut surfaces of the explants and these compounds may lead to death of the explants (Ahmad 2013). Safwat *et al*. (2015) reported that phenolic exudation of explants is overcome by adding antioxidants like citric acid (150mgL^{-1}) and ascorbic acid (150mgL^{-1}) to the culture medium and results in inhibition of browning or blackening in the culture medium. Time taken for shoot initiation also varies and it was further noted that slow growth of the explants as it belongs to cactus family. Lowest time taken for shoot initiation (23 days) was recorded in MS media supplemented with 2mgL^{-1} of BAP and it was highest (45 days) in MS basal (control) (Table 3).

Shoots longer than 1.5cm was transferred into freshly prepared MS medium and it is usually carried out for every two weeks and it was noted that fungal contamination takes place after 1 week of subculturing which reduced the survival percentage. The addition of cytokinin in the culture medium breaks the dormancy and activates the areoles under *in vitro* conditions and improves the proliferation rate has been reported in various cactus species like Opuntia (Estrada *et al*., 2008). Multiple shoot formation takes place when the explants were cultured on multiplication medium containing different combinations of growth regulators. Induction of multiple shoots from different explants of cacti has been reported earlier with respect to response of different cytokinins such as BA (Dahanayake *et al*., 2011). In the present study BAP was used for multiple shoot induction.

Highest number of multiple shoots (7) was recorded in MS media supplemented with 7mgL^{-1} of BAP + 1mgL^{-1} kinetin and least was observed in MS media supplemented with 3mgL^{-1} of BAP (Table 4). Subculture was carried out at regular intervals of every 20 days and shoot length was recorded during 60 to 70 days after inoculation till it attains the maximum length. Shoot length was recorded highest (3.5 cm) in MS media supplemented with 7mgL^{-1} of BAP + 1mgL^{-1} kinetin and it was lowest (2.7cm) in MS media supplemented with 3mgL^{-1} of BAP (Table 4).

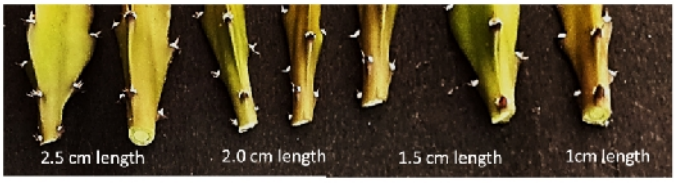

Fig. 1. Young shoot tips of different sizes taken as explants for *in vitro* propagation of dragon fruit.

Fig. 2. Multiple shoot initiation from explants. (A & B) MS medium containing 2mgL^{-1} BAP. (C) MS medium containing 3mgL^{-1} BAP and 1mgL^{-1} Kinetin. (D) MS medium containing 4mgL^{-1} BAP and 1mgL^{-1} Kinetin. (E) MS medium containing 5mgL^{-1} BAP and 1mgL^{-1} Kinetin. (F) MS medium containing 7mgL^{-1} BAP and 1mgL^{-1} Kinetin.

CONCLUSION

In vitro propagation is the best alternative method in dragon fruit for mass multiplication to produce disease free and true to type planting material**.** Based on the present study, the explant of 2cm in length, with 4 to 5 nodes, showed better response of survival after pretreatment with 0.1% Bavistin and surface sterilization with $HgCl_2$ (0.1% for 3 min). MS medium supplemented with $2mgL^{-1}$ of BAP exhibited highest response for shoot proliferation. However, the results revealed that MS medium fortified with $7mgL^{-1}$ BAP + 1 mgL^{-1} Kinetin was found to be the best in terms of earlier response of shoot multiplication, highest number of multiple shoots and maximum shoot length. Hence this protocol could be utilized for mass multiplication of dragon fruit under *in vitro* conditions.

FUTURE SCOPE

Future studies need to be carried out using different explants in order to improve the multiplication rate for further breeding programmes.

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Conflict of Interest. None.

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