# Rapid In Vitro Multiplication of *Hylocereus undatus* (Haw.) Britton & Rose

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ABSTRACT

*Hylocereus undatus* (Dragon fruit), a climbing cactus, belongs to the family Cactaceae. It is a widely accepted fruit crop. Limitations of large-scale propagation can be overcome through *in vitro* propagation techniques. In the present study, we investigated the possibility of regeneration of dragon fruit through direct and indirect organogenesis from the hypocotyl explants obtained from its *in vitro* germinated seedlings. The highest callus induction and proliferation was obtained in MS medium supplemented with 2.5 mg/l 2,4-D. Maximum number of multiple shoots (18.1±0.4) from calli was recorded on MS media supplemented with 1 mg/l BAP + 1.5 mg/l NAA. The highest rate of adventitious shoot formation (direct organogenesis) was in MS + 1.5 mg/l BAP. Regenerated shoots developed healthy roots in MS + 3 mg/l NAA. Rooted plantlets were acclimatized successfully in pots containing sand and cocopeat (1:1).

**Key words: Callus regeneration, Direct organogenesis, Dragon fruit**

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## Introduction

*Hylocereus undatus* (Haw.) Britton & Rose commonly called Pitaya is a perennial epiphytic climbing cactus with triangular fleshy, jointed stems. It is also known as Dragon fruit in Asia, because of its bright red skin with green overlapping fins covering the fruit (Zee et al., 2004). In Southeast Asia, both fruits and flowers are consumed, the fruit being more important. The fruit has a white, delicately sweet pulp, and numerous, edible, black seeds embedded within the pulp. Peeled fruit slices are amongst the favoured post dinner deserts. *Hylocereus undatus* have drawn a lot of attention in recent years because of its economic and medicinal properties. Dragon fruit has a creamy pulp with delicate aroma, and its nutritional properties make the fruit an attractive raw material for different kinds of ice creams, jam, jelly, soft drinks (Islam et al., 2012; Dam, 2013) and cookies (Ho & Latif, 2016). Dragon fruit serves as a source of alternative medicine, because of the presence of antioxidant agents in fruit pulp and peel (Mahattanatawee et al., 2006). Dragon fruit is helpful in reducing blood sugar level because of its anti-diabetic properties (Sudha et al., 2017). Dragon fruit extract is beneficial for stem cell proliferation and differentiation, essential for bone tissues (Hartono et al., 2020). The low calorific value and the presence of micronutrients in fruit pulp is a great benefit for use in food, especially in low caloric diet (Jeronimo et al., 2015). Seed oil of Dragon fruit contains high level of essential fatty acids (Ariffin et al., 2009). Pitaya peels are often discarded during processing, but the presence of dietary fiber in peels increases the intestinal health (Zhuang et al., 2012).

The large-scale multiplication of *H. undatus* is possible using stem cuttings and seeds. Vegetative propagation of dragon fruit is difficult because seed derived plants have long juvenile period and delayed fruiting (Bellec et al., 2006). Standardized length of planting material is essential for proper growth and for adapting diverse environmental conditions (Kakade et al., 2019). In vitro tissue culture technique could enable highly efficient multiplication of plants in relatively short time using little starting material. Plant tissue culture is being widely used for large scale production of plants within a short time. Moreover, in vitro propagation techniques have importance in plant propagation, disease elimination, crop improvement and production of secondary metabolites (Giusti et al., 2002; Goncalves & Romano, 2013).

Several researchers have reported rapid, highly efficient micropropagation methods for *H. undatus* which shows different effects on plant regeneration (Yang et al., 2011; Fan et al., 2013; Yassen, 2002). The present study was conducted to determine the influence of different concentrations of plant growth hormones for optimum shoot and root proliferation through direct and indirect organogenesis.

## Materials and Methods

### *Seed germination and preparation of explants*

Seeds of red Dragon fruit collected from fresh ripe fruit, which were purchased from the local market, were used for raising the in vitro seedlings. Seeds were extracted from fleshy pulp using a clean spoon and soaked overnight in water and kept in a drier to remove the fleshy covering. Then the seeds were washed under tap water for 20 minutes followed by three times washing using liquid soap for 15 minutes. Further sterilizations were carried out in a laminar flow cabinet. Then the seeds were surface sterilized thoroughly in 70% alcohol for 5 minutes and washed in distilled water containing 2-3 drops of Tween-20 for 20 minutes. Sterilized seeds were then properly washed with sterile distilled water for 5 or 6 times. Surface sterilized seeds were inoculated in half strength MS medium (Murashige & Skoog, 1962) containing 30g/l sucrose without plant hormones. All culture media used in the study were solidified with 0.8% agar and their pH was adjusted to 5.8 with 1N NaOH and 1N HCL prior to autoclaving at 121°C for 20 minutes. The medium was then dispensed to culture vessels; 30 ml to each bottle and then autoclaved along with other instruments required for aseptic transfer of explants. Seeds were inoculated in media and bottles were tightly capped after quick flaming and sealed with parafilm to avoid contaminations. All the cultures were maintained in a growth room maintained at a temperature of 22± 2°C and 16 h photoperiod.

### *Callus induction and regeneration*

Healthy *in vitro* germinated seedlings were randomly selected for the excision of 1.5 cm long hypocotyl explants, which were cultured in half strength MS medium fortified with 2,4-dichlorophenoxyacetic acid (2,4-D) and Naphthalene-acetic acid (NAA) alone and in combination. Callus induction and proliferation were tested in same media composition. Intensity, color, and nature of callus were recorded. After subculturing at regular intervals well proliferated calli were excised and transferred to regeneration media which contained different combinations of auxin and cytokinin for shoot development.

### *Direct shoot organogenesis*

As for raising the callus, hypocotyl segments (1.5 cm long) were used as explants for direct organogenesis. Explants were inoculated aseptically in MS medium supplemented with different concentration of BAP (0.5 mg/l – 2.5 mg/l). The cultures were sub-cultured at regular intervals by transferring to fresh medium each time. Optimum regeneration ability of shoots was determined. Number of shoots and length of shoots were recorded after 30 days of culture. Elongated shoots were excised and transferred to MS medium supplemented with NAA. For rooting, five concentrations of NAA were used (1, 1.5, 2, 2.5 and 3 mg/l). Until the induction of roots, cultures were sub-cultured regularly in fresh medium and maintained in controlled conditions. Mean number of roots and average length of roots were recorded after four weeks. For each treatment, three replicates were used, and all the experiments were repeated twice. Statistical analyses were conducted using MS Excel.

**Acclimatization of plantlets:** After four weeks of culture, the rooted plantlets were carefully removed from the rooting media and cleaned using sterile distilled water to remove the excess media present on the root surface. The plantlets were transferred to pots containing an autoclaved mixture of cocopeat and sand in the ratio of 1:1. The transferred plantlets were covered with polythene sheets to maintain humidity.

## Results

### *Mass multiplication via callus*

In the present study, healthy stem segments were selected from in vitro germinated seedlings as explants. For callus induction, healthy stem segments with length of 1.5 cm were cultured in half strength MS medium supplemented with 2,4-D, 2,4-D + NAA and NAA (Table 1). Explants start to develop callus within two weeks. Callus formation began from the basal part of explant. Callus formation was observed in all treatments. Callus induction failed in MS medium without a growth regulator. Maximum callusing was obtained in medium supplemented with 2.5 mg/l 2,4-D and the calli were cream- coloured and friable in nature (Fig. 1A). Different combinations and concentrations of phytohormones yielded different types of calli, in terms of color and texture. Explants grown in media with 1.0 mg/l 2,4-D and 1 mg/l NAA also developed friable green callus. Poor callus formation was observed in other treatments.

Well proliferated cream- friable and green-friable callus were transferred to regeneration media. Calli were excised and transferred on to half strength MS medium supplemented with various concentrations of BAP alone or in combination with NAA (Table 2) for

**Table 1. Callusing response observed after 30 days of culture of hypocotyl explants in different media.**

| Combination of phytohormones (mg/l) | Response percentage (%) | Intensity of callus formation | color | Texture |
|---|---|---|---|---|
| 2.0 2,4-D | 60 | + + | Cream | Friable |
| 2.5 2,4-D | 100 | + + + + | Cream | Friable |
| 3.0 2,4-D | 70 | + + | Cream | Friable |
| 1.5 NAA | 40 | + | White | Friable |
| 2.0 NAA | 50 | + | White | Friable |
| 2.5 NAA | 60 | + + | White | Friable |
| 3.0 NAA | 60 | + + | Cream | Semi friable |
| 0.5 2,4-D + 0.5 NAA | 60 | + + | Cream | Semi friable |
| 2.5 2,4-D + 0.5 NAA | 80 | + + + | Greenish | Friable |
| 3.0 2,4-D + 1.0 NAA | 70 | + + + | Greenish | Friable |
| 1.0 2,4-D + 1.0 NAA | 100 | + + + + | Greenish | Friable |

*Profuse: ++++, Moderate +++, Poor: +, No response: -

regeneration. Shoot generation was observed from the cream friable calli, whereas green friable calli failed to regenerate. The highest regeneration frequency was in the medium containing 1mg/l BAP and 1.5 mg/l NAA, with average number of shoots 18.1±0.48 (Fig. 1B). Multiple shoots were developed from callus in the medium fortified with low concentration of BAP also. Differences in length of multiple shoots were observed in both kinds of media. Multiple shoots developed in the medium containing BAP and NAA were comparatively short with an average length of 3.98±0.15 (Fig. 1C). The medium fortified with BAP and NAA induces root formation along with shoot developments. Root number and root length showed differences among various concentrations of NAA and BAP (data not shown here). Light, brown-colored long roots (6 roots per plant) were formed in the medium containing 1.5 mg/ml BAP and 1.5 mg/ml NAA (Fig. 1D).

**Table 2. Regeneration of shoots from the calli cultured in media supplemented with BAP and NAA, after 55 days of inoculation.**

| Combination of phytohormones (mg/l) | Average No. of shoots per callus | Average length of shoots (cm) |
|---|---|---|
| 0.5 BAP | 8.8 ±0.48 | 5.20±0.17 |
| 1.0 BAP | 9.1±0.38 | 6.54±0.04 |
| 1.5 BAP | 6.7±0.21 | 6.28±0.15 |
| 2.0 BAP | 11.3±0.57 | 4.87±0.04 |
| 0.5 BAP + 1.0 NAA | 9.4±0.33 | 3.21±0.17 |
| 1.0 BAP + 1.5 NAA | 18.1±0.48 | 3.15±0.21 |
| 1.5 BAP + 1.5 NAA | 10.1±0.63 | 3.98±0.15 |
| 2.0 BAP + 1.5 NAA | 6.7±0.66 | 3.64±0.04 |

Each value represents mean ±SE.

### *Direct organogenesis*

Regeneration ability of hypocotyl segments was investigated in half strength MS medium containing different concentrations of BAP (0.5 mg/l -2.5 mg/l). Adventitious shoots started to develop within two weeks. Explants cultured in the medium supplemented with 1.5 mg/l BAP regenerated maximum number of shoots (10.1 ±0.3) (Fig. 1E). Multiple adventitious, thick, and fleshy shoots were developed after 10 days of culture. Good spine formation was observed in all developing shoots (Fig. 1F). We found that the maximum number of shoots were produced in basal MS media with low concentration of BAP.

Well elongated shoots (2 to 3 cm) were excised and transferred to MS medium supplemented with NAA (1 mg/l to 3 mg/l). Number of roots per explant and length of roots was measured. White, healthy roots developed in all the media (Fig. 1H). Among the different concentrations, a medium with 3 mg/l NAA induced the maximum number of roots (4.4±0.17) with length of 3.16±0.17 within three weeks. Regenerated adventitious shoots also developed roots in the rooting media. Here too, good spine formation and bending of shoots was observed along with root formation (Fig. 1G). Well rooted plants were taken out and transferred to soil followed by acclimatization stages.

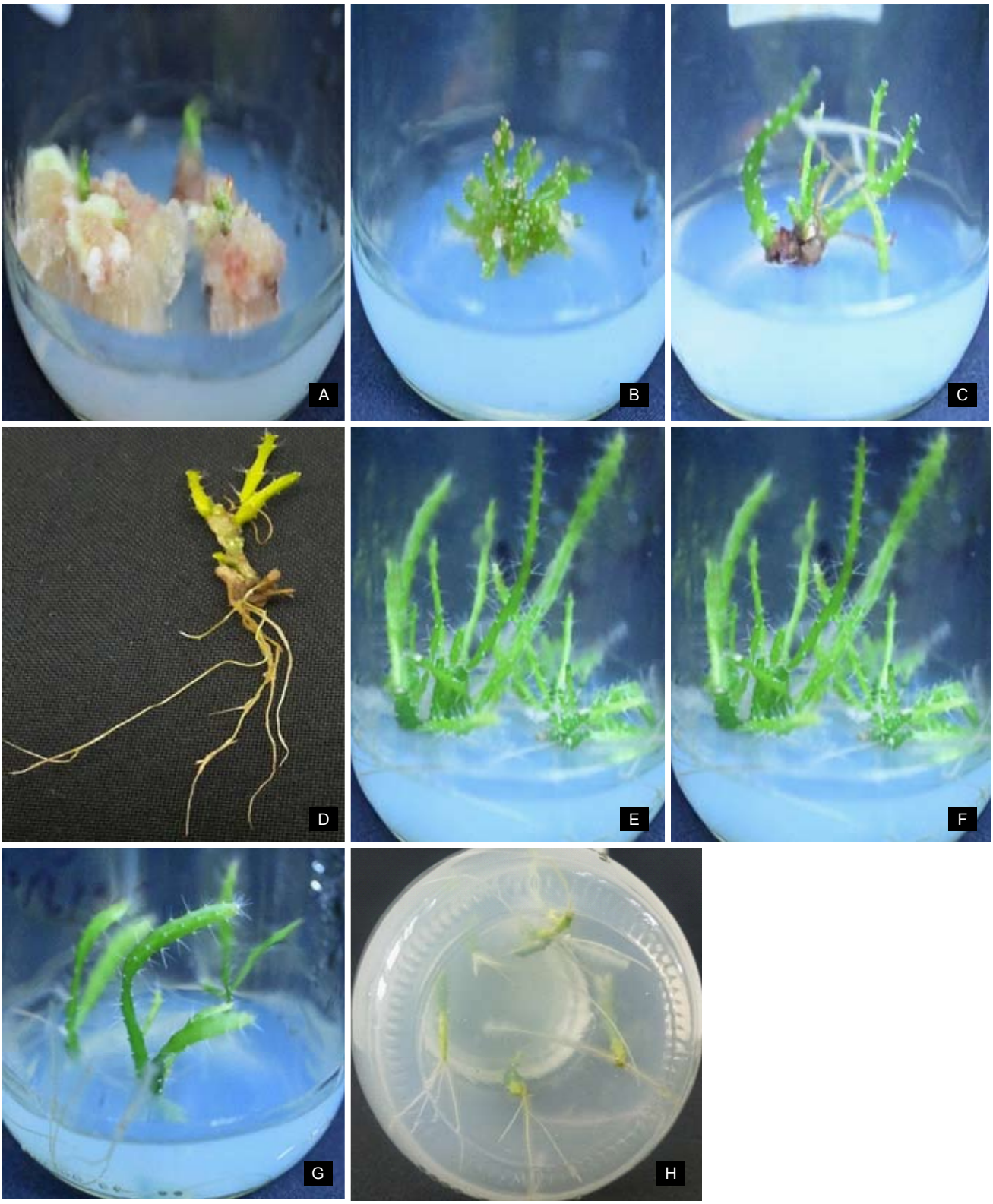


**Figure 1. (A-D) *In Vitro* regeneration through indirect organogenesis using stem explants. (A). yellow friable callus induction on MS +2.5 mg/l 2,4-D; (B) multiple shoot regeneration on MS + 1 mg/l BAP + 1.5 mg/l NAA; (C) Multiple shoot regeneration from callus on 1.5 mg/l BAP; (D) Brown-coloured root formation on shoots on MS +1.5 mg/l BAP + 1.5 NAA; (E-H) Regeneration of explants via direct organogenesis; (E) Formation of adventitious shoots from stem segment on MS+ 1.5 mg/l BAP; (F, G) Spine development in shoots; (H) Root formation on MS+3 mg/l NAA**

## Discussion

Limitations of large-scale propagation can be overcome through in vitro culture methods. In the present study, mass multiplication of *H. undatus* is reported. Plant growth regulators play an important role in the growth and development of plant cells. The combination of auxins and cytokinin has a major role in the proliferation and development of callus (Kulus, 2020). Previous studies show that stem cuttings of adult individuals of *H. undatus* have high regeneration ability (Dahanayake and Ranawake, 2011). Here we successfully used stem segments of seedlings germinated *in vitro.*

In this study good callus formation from stem segments of seedlings was achieved on medium fortified with 2,4-D. These results agree with previous findings that auxins play an important role in callus formation and proliferation (Huh et al., 2017). 2,4-D is a synthetic auxin hormone that is used in callus culture due to the stimulation of the de-differentiation process and organogenesis (Tahir et al., 2011). 2,4-D works effectively at low concentrations, this can be seen from our results. A study by Thinesh and Seran (2015) shows that the combination of TDZ and BAP is suitable for callus formation from *H. undatus*. but interestingly, the combination of phytohormones for callogenesis and shoot regeneration from callus was entirely different from our findings. Rumiyati et al. (2017) obtained a good callus formation of dragon fruit in a medium containing 2,4-D. Generally, NAA and 2,4-D alone or in combination were found to be the best plant growth regulators for callus induction. Formation of adventitious shoots from callus in the presence of auxin and cytokinin has been reported in several other medicinal plants (Sen et al., 2014; Quin et al., 2017).

In our study, a combination of BAP and NAA resulted in good shoot regeneration. As per the studies on *H. undatus* carried out by Kasim et al. (2019), MS medium fortified with 3 mg/l BAP and 0.5 mg/l NAA exhibited highest response for shoot regeneration within 70 days. In the present study more proliferation and regeneration of shoots occurred in MS medium fortified with 1 mg/l BAP and 1.5 mg/l NAA within 50 days. Lower concentration of cytokinin promoted active growth of healthy shoots without abnormalities in dragon fruit (Vinas et al., 2012). In several plant species, cytokinin combinations increase shoot multiplication (Torres-Silva et al., 2018: Suman et al., 2017).

A well-developed root system is the key step for vegetative propagation. The application of auxins to in vitro cultures promotes root formation and enhances rooting rate (Skoog & Miller, 1957; Shimomura & Fujihara, 1980). In the present study, the type and concentration of auxin influenced the average number of roots per explant and mean length of roots. In our experiment we observed rooting along with shoot formation in the presence of lower BAP and NAA concentrations. The formation of roots from in vitro shoots of *H. undatus* promoted by auxin has been reported earlier (Dahanayake & Ranawake, 2011).

In the present study, we report mass multiplication of *Hylocereus undatus* through direct organogenesis. We also developed a protocol for indirect organogenesis using calli derived from hypocotyl explants for large scale propagation. Our study showed that low concentration of auxin and cytokinin yields the best results for shoot and root proliferation within a less period of time

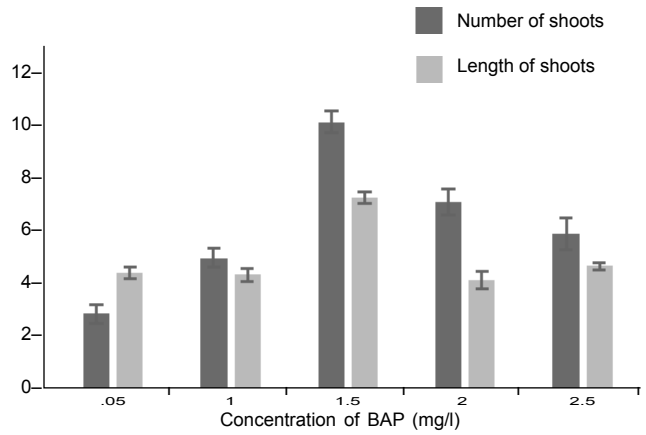


**Figure 2. Effect of BAP on direct shoot regeneration from the explants.**

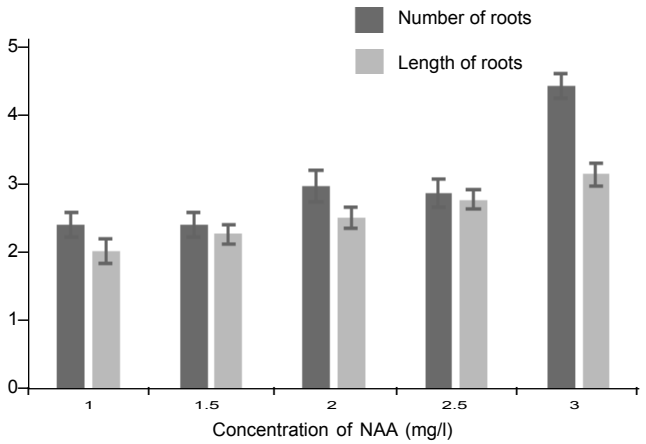


**Figure 3. Effect of NAA on root regeneration from the micro-shoots.**

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