# *In vitro* Shoot Multiplication in *Hyoscyamus niger* L. from Nodal Segments

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## Abstract
*Hyoscyamus niger* contains tropane alkaloids such as scopolamine and hyoscyamine which are highly important for medicinal purposes. The present objective was to develop an efficient protocol for shoot multiplication of *Hyoscyamus niger* L. from nodal segments of *in vitro* raised seedlings. Seed germination was recorded on MS and Gamborg$B_5$media however 100% germination was recorded on Murashige and Skoog (MS) full strength media. Among the combinations of hormones tested, good response of shoot proliferation with maximum shoot number and maximum shoot length was observed from nodal segments on MS + TDZ [14 Micromoles (µM)] + IBA (3.5µM) after 32 days of culture.
**Keywords.** Plant tissue culture, *Hyoscyamus niger*, nodes, multiple shoots

## Introduction
Majority of the world's population depend on medicinal plants as they are the main and essential life saving drugs (Khan *et al.*, 2009). Plant derived medicines constitute a substantial component of present day human health systems both in developed as well as in developing countries (Gomez-Galera *et al.*, 2007). These medicinally important plants produce secondary metabolites which have very high economic importance as drugs, fragrances, pigments, food additives and pesticides etc (Khan *et al.*, 2009). So a large variety of techniques like, DNA manipulation, genetic engineering, transformation, fermentation, and plant tissue culture for conservation, improvement in secondary metabolite and development of transgenic plants have been developed.Hence plant tissue culture techniques can be the source of alternative method for continuous multiplication of plantlet stocks for large scale drug production, field conservation, mass propagation and crop improvement as well (Baraket and El-Sammak, 2011).

Keeping the importance of biotechnological tools in consideration the current study focuses on shoot proliferation of under *in vitro* conditions of an important medicinal plant namely *Hyoscyamus niger* L. This plant is considered to be a rich repository of alkaloids.It is commonly known as black henbane, devil's eye, poison tobacco, stinking nightshade, (Heiser.,1969) and Bazer bang in Kashmiri. This species is distributed in China, Afghanistan, India, Japan, Korea, South-West Asia, North Africa and throughout Europe (Sajeli *et al.*, 2006) at an altitude of 1500 m and 2300 m. *H. niger* is classified as threatened non-endemic plant of Kashmir (Dar *et al.*, 2002) and different threat status has been assigned to this herb by different authorities, viz-a-viz; Low risk-near threatened (Ved and Tandon, 1998), rare (Dar and Naqshi, 2001) or vulnerable (Kumar *et al.*, 2011).The plant is almost 1-1/2 ft in height and its leaves have granular hairy surface, with greenish grey colour (Khory and Katrak, 1985). The flowers are almost stalkless and the bell-shaped corolla is yellowish with purple veins. The calyx enlarges as the fruit develops and surrounds many seeded capsule

(Heiser, 1969). It produces enormous amount of seeds ranging from ten thousand to half a million seeds and these are highly poisonous to children as they contain hyoscyamine, fixed fatty oil, an empyreumatic oil and ash (Khory and Katrak, 1985). *Hyoscyamus* plants differ from other Solanaceous genera by having a fruit composed of a thorn less seed capsule with a cover (Strauss, 1989). *Hyoscyamus* species are rich sources of tropane alkaloids, mainly hyoscyamine and scopolamine (Supria, 1998). *H. niger* is used for the treatment of various illnesses such as ear and eye inflammation, rheumatism, treatment of ulcers, cough, motion sickness, asthma, rabies, fevers, bronchitis, renal colic, and spasm (Hong *et al*., 2012) and also has spasmolytic, antiasthmatic, anticholinergic, narcotic and anaesthetic properties (Roddick, 1991). Scopolamine is used for the treatment of abdominal pain associated with cramps induced by gastrointestinal spasms (Woo *et al.*, 1995).Due to increasing commertial demand and high rate of explitation the estamisment of *in vitro*protocol for large scale mass propagation is neededd. Thus the current study aims at standardizing a protocol for *in vitro*shoot multiplication of *Hyoscyamus niger* L. using nodal explants.

## Material and Methods

Seeds of *Hyoscyamus niger* were procured from the CIMAP, Lucknow. *In vitro* raised nodes, of *Hyoscyamus niger* L. were used as explants. The seeds were first washed with detergent (labolene and extran 0.5%) followed by the drops of tween-20 (surfactant). The already cleaned seeds were subjected to chemical sterilization using sodium hypochlorite (0.5, .10, 0.15 and 2% (v/v)) and mercuric chloride (HgCl2) 0.1,0.02,0.03 and 0.4 (v/v) for different times periods finally seeds were washed thoroughly several times with sterile distilled water.

### *Selection of nutrient media*

The success in cell, tissue and organ culture is related to the selection or development of a suitable culture medium as no single medium supports the growth of all tissues. Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice, nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to a particular species. Media compositions are, therefore, formulated considering specific requirements of a particular culture system (Razdan, 2016). The nutrient media selected for carrying out present investigation were Murashige and Skoog medium (MS medium) (full and half strength) and Gamborg$B_5$ medium.

### *Seed germination*

Seeds were efficiently surface sterilized with mercuric chloride (0.2% v/v) for 20 minutes, followed by six washes with autoclaved distilled water. After that seeds were cultured on agar supported hormone free MS full strength, MS half strength and Gamborg $B_5$ media.

### *Shoot differentiation*

Nodes from the*in vitro* germinated seeds were transferred onto MS media supplemented with different hormonal concentrations [6-benzyl amino purine (BAP), BAP+ NAA, Thidiazuron (TDZ), TDZ + NAA, TDZ +IAA, TDZ+IBA] sucrose and gelling agents. Shoot differentiation and elongation were carried out under cool fluorescent tubes at a 16 hour photoperiod regime with light intensity of 1500 – 3000 lux at a constant temperature of 25±3ºC. Relative humidity between 60-70% was maintained.

## Results

### *Seed germination*

Seed sterilization of *H. niger*was obtained on $HgCl_2$ (0.2 %) for 20 min with 90% sterilization and 100 % explant survival rate. Sterilized seeds were inoculated on MS full and half strength as well and on Gamborg$B_5$ media (**Figure1**). However, encouraging results in terms of seed germination i.e. 100% were obtained on MS full strength medium only after 3 weeks of culture period (**Figure 2**).

### *Shoot multiplication*

Aseptically *in vitro* raised nodal segments were cultured on MS media supplemented with different concentrations of BAP (0-16 µM) alone as well as with combination with NAA. Nodes did not show any response on MS basal media. The indirect multiple shoot regeneration with Brownish Nodular Callus (BNC) was observed on all the concentrations of BAP (1µM to 16µM). However, MS medium supplemented with BAP (8 µM) resulted in maximum average increase in shoot number (5.4) and shoot length (2.1 cm) .The shoot number and shoot length decreased with further increase in concentration of BAP (**Table 1**). However, it was observed BAP in combination with NAA sharply decreased percentage of shoot formation as well as shoot length. The maximum average shoot number (4.5) and 2.2 cm shoot length was obtained on BAP (4µM) + NAA (1µM) (Table 1).Further increase in concentration of BAP /NAA showed decreasing trend of shoot multiplication.

Simultaneously, TDZ alone as well as in combination with NAA, IAA, IBA for observing the better growth response and results obtained are overwhelming. Brownish Nodular Callus (BNC) at the base of explants and indirect multiple shoot regeneration followed by their elongation on the same medium were seen on all the concentrations of TDZ. As wilting of leaves was observed in individually supplemented TDZ media, then we have used the TDZ in combination with other hormones. Among the various combinations, MS + TDZ (14 µM) + IBA (3.5µM) showed maximum average shoot number (13.0) and shoot length (3.1cm) with healthy leaves **(Figure 3).**

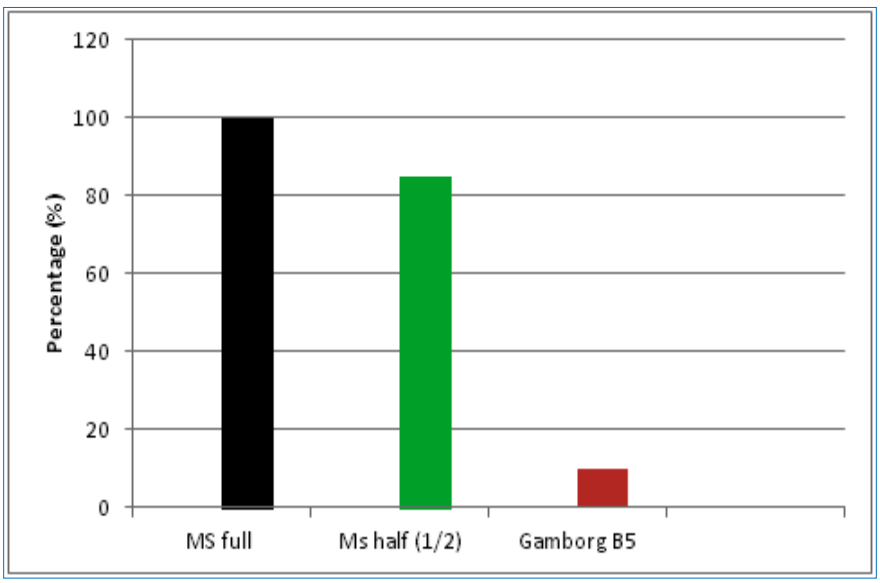


**Figure 1. Seed germination (%) of *Hyoscyamus niger*on different media.**

**Table 1: Effect of plant growth hormones (BAP/TDZ/NAA/IAA/IBA) on shoot multiplication of *H. niger*from *in vitro* raised nodes**

| Treatments | Shoot-No | Shoot length (cm) | Shooting response (%) | Callus formation |
|---|---|---|---|---|
| Control (MS basal) | - | - | - | - |
| BAP(1µM) | $^{b}$3.5± 0.51 | $^{a}$2.0±0.78 | 100 | ++ |
| BAP(4 µM) | $^{c}$4.2± 0.60 | $^{a}$2.0±0.56 | 100 | ++ |
| BAP(8 µM) | $^{d}$5.4± 0.26 | $^{a}$2.1±0.78 | 100 | + |
| BAP(12 µM) | $^{c}$4.2± 0.43 | $^{a}$2.1±0.74 | 80 | + |
| BAP(16 µM) | $^{bc}$4.0± 0.41 | $^{a}$2.0±0.34 | 80 | ++ |
| TDZ (1 µM) | $^{e}$7.8± 0.21 | $^{a}$2.4±0.46 | 100 | +++ |
| TDZ (4 µM) | $^{ef}$8.1± 0.59 | $^{a}$2.7±0.85 | 100 | +++ |
| TDZ (8 µM) | $^{f}$8.5± 0.48 | $^{a}$2.7±0.36 | 100 | +++ |
| TDZ (12 µM) | $^{f}$8.9± 0.39 | $^{b}$3.0±0.64 | 100 | +++ |
| TDZ (16 µM) | $^{g}$10.4± 0.12 | $^{b}$3.1±0.86 | 100 | +++ |
| TDZ (20µM) | $^{ef}$8.0± 0.62 | $^{ab}$2.8±0.46 | 100 | ++ |
| BAP(1µM)+NAA(1µM) | $^{a}$2.1± 0.51 | $^{a}$2.1±0.63 | 70 | + |
| BAP(3 µM)+NAA(1 µM) | $^{bc}$4.1± 0.39 | $^{a}$2.1±0.63 | 70 | + |
| BAP(4 µM)+NAA(1 µM) | $^{c}$4.5± 0.50 | $^{a}$2.1±0.64 | 80 | + |
| BAP(5 µM)+NAA(1 µM) | $^{bc}$4.0± 0.36 | $^{a}$2.2±0.74 | 70 | + |
| TDZ (1 µM) +NAA(1 µM) | $^{b}$3.2± 0.45 | $^{a}$2.2±0.74 | 80 | + |
| TDZ (3 µM) +NAA(1 µM) | $^{c}$4.5± 0.23 | $^{a}$2.2±0.37 | 80 | ++ |
| TDZ (4 µM) +NAA(1 µM) | $^{d}$5.4± 0.37 | $^{a}$2.3±0.52 | 80 | ++ |
| TDZ (5 µM) +NAA(1 µM) | $^{d}$5.0± 0.35 | $^{a}$2.3±0.63 | 80 | ++ |
| TDZ (1 µM) +IAA(1 µM) | $^{e}$7.0± 0.65 | $^{b}$2.1±0.86 | 90 | ++ |
| TDZ (4 µM) +IAA(1.5 µM) | $^{e}$7.4± 0.39 | $^{b}$2.2±0.83 | 90 | ++ |
| TDZ (8 µM) +IAA(2 µM) | $^{e}$7.4± 0.35 | $^{b}$2.2±0.66 | 90 | ++ |
| TDZ (10µM)+IAA(2.5 µM) | $^{f}$8.3± 0.15 | $^{b}$3.1±0.46 | 90 | ++ |
| TDZ (12 µM) +IAA(3 µM) | $^{f}$8.0± 0.39 | $^{b}$2.1±0.76 | 90 | ++ |
| TDZ (14µM)+IAA(3.5 µM) | $^{f}$8.9± 0.85 | $^{ab}$2.9±0.86 | 100 | ++ |
| TDZ (1 µM) +IBA(1 µM) | $^{f}$8.0± 0.36 | $^{ab}$2.9±0.86 | 100 | ++ |
| TDZ (4 µM) +IBA(1.5 µM) | $^{f}$8.7± 0.35 | $^{ab}$2.9±0.86 | 100 | ++ |
| TDZ (8 µM) +IBA(2 µM) | $^{g}$9.1± 0.38 | $^{b}$2.9±0.86 | 100 | +++ |
| TDZ (10µM) +IBA(2.5 µM) | $^{g}$9.6± 0.35 | $^{b}$3.0±0.86 | 100 | +++ |
| TDZ (12 µM) +IBA(3 µM) | $^{h}$11.9± 0.75 | $^{b}$3.0±0.86 | 100 | +++ |
| TDZ (14µM) +IBA(3.5 µM) | $^{h}$13.0± 0.35 | $^{b}$3.1±0.16 | 100 | +++ |
| TDZ (16 µM) +IBA(4 µM) | $^{g}$10.0± 0.55 | $^{b}$3.1±0.86 | 100 | +++ |
| TDZ (20µM) +IBA(4.5 µM) | $^{e}$7.0± 0.35 | $^{b}$2.8±0.86 | 100 | +++ |

**Values are represented as mean±SD (n=10), Data was analyzed by ANOVA using Duncan's multiple range test (SPSS17.0); the values with different superscript along the columns are statically significant at P<0.005. Data scored after 8 weeks of culture period, + (low callus); (++) moderate callus; (+++) intense callus.*

**Figure 2: Seed germination of *H.niger* on MS media full strength**

**Figure 3: Shoot multiplication from *in vitro* nodes on MS + TDZ (14 µM) + IBA (3.5µM)**

**Discussion**

Micropropogation is a technique to grow, regenerate and to multiply plants in aseptic or sterile conditions (Hartmann *et al.,* 2011). It has been a major challenge since long to grow contamination free *in vitro* plantlets using field grown material as explants (Webster *et al.,* 2003). In present study seed germination and complete seedling formation was recorded on MS basal media. Similar results were achieved by Uranbey (2005) in *Hyoscyamus niger,* Aminnejad*et al,* (2015) in *H. reticulatus,* Keng *et al.* (2009) in *H. mutatus* and Sen and Sharma (1991) using MS basal media.

Regeneration of plantlets by cell, tissue and organ culture is a major goal of plant tissue culture and great deal of work on regeneration has been devoted in finding equilibrium between the components of the medium. Nodes were taken as ex-plants, and showed shoot formation on MS media supplemented with different phytohormones. Study revealed that MS media supplemented with MS + TDZ (14 µM) + IBA (3.5µM) showed maximum average shoot number (13.0) and shoot length (3.1cm) was obtained. However, BAP was found to be less effective in shoot regeneration compared with TDZ. It has been indicated that TDZ was effective than BAP on shoot regeneration in all hypocotyls, cotyledon, and stem explants of henbane (Uranbey, 2005).TDZ has been considered to be more powerful than most commonly used cytokinins (Huetteman and Preece, 1993) as a plant growth regulator to stimulate high rate of axillary shoot regeneration in woody plants (Malik and Saxena, 1992). It also releases the lateral bud dormancy and stimulates shoot formation in wide variety of plant species (Malik and Saxena, 1992). Similar results were recorded Babaei *et al.,* 2014 who reported the most effective concentration was combination of 0.5mg L−1 TDZ and 0.25 IBA mg L−1 than TDZ alone for shoot regeneration of shoots in *Curculigo latifolia* under *in vitro* conditions. Uranbey (2005) reported in *H. niger* using hypocotyl, cotyledon and stem explants TDZ was more effective than BAP. Thomas, 2007 reported in *Curculigoorchioides*that there was an increase on percentage of explants producing shoots and shoot numbers with 1.5mg L−1 of TDZ at all levels of

IBA.TDZ was reported to be more successful than BA and KIN for shoot regeneration in meadowsweet (Yildirim and Turker, 2009).

**Conclusion**

An efficient protocol for *in vitro* shoot multiplication of *H.niger* from nodal explants was developed. MS full strength medium has given 100% seed germination, while maximum shoot multiplication from nodal explants was observed from the combinationof TDZ (14 µM) + IBA (3.5µM). The developed protocol on micropropogation of *H. niger* can be utilized for its mass production.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

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