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Effect of 6-Benzyladenine, Kinetin and Thidiazuron on *in Vitro* Shoot Proliferation of *Hyoscyamus niger* L.

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ABSTRACT

Hyoscyamus niger is an important medicinal plant belonging to the Solanaceae family, commonly known as henbane and locally as *Bazir bangh*. A study was undertaken to investigate the effects of different plant growth regulators (PGRs) at various concentrations on shoot proliferation from shoot tip explants. Full-strength Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyladenine (BA), kinetin (Kn) and thidiazuron (TDZ) were tested. Amongst the PGRs tested, BA at 12.5 µM showed the best shoot proliferation and highest number of indirect multiple shoots while TDZ at 10 µM formed intense disorganized friable light-greenish callus only; there was no response with Kn. *In vitro* shoots were separated and rooted under varying concentrations of different auxins viz. indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA). Best rooting response was recorded with 4.0 µM IBA and 2.5 µM NAA. However, no response was noted with any concentration of IAA. Plantlets transferred to field conditions showed 50% survival.

Keywords: Bazir bangh, multiple shoot, plantlet
Abbreviations: BA, 6-benzyladenine; **IBA**, indole-3-butyric acid; **IAA**, indole-3-acetic acid; **NAA**, α-naphthalene acetic acid; **Kn**, kinetin; **TDZ**, thidiazuron; **MS**, Murashige and Skoog medium; **PGR**, plant growth regulator

INTRODUCTION

Hyoscyamus niger, commonly known as henbane and locally known as *Bazir bangh*, belongs to the Solanaceae family. The original range of distribution of *H. niger* embraced Europe, Western and Central Asia and North Africa (Stary and Berger 1990). In India, it occurs in the Western Himalayas from Kashmir to Garhwal at an altitude of about 1500-3000 m a.s.l. In the Kashmir valley, its distribution is rare in certain areas such as Karnah, Gurez and Lolab whereas in Gulmarg and Sonamarg it is found occasionally (Kaul 1997). *H. niger* is classified as a threatened non-endemic plant of Kashmir (Dar *et al*. 2002) and a threat status of low risk – near threatened has been assigned to this herb by Ved and Tandon (1998) and rare by Dar and Naqshi (2001). Principal alkaloids are hyoscyamine and hyoscine. Traces of atropine and scopolamine are also present. Roots contain on average 0.16%, leaves 0.045-0.08%, flowering tops 0.07-0.1% and seeds 0.06-0.1% of alkaloids (Kaul 1997). The dried leaves and flowering tops collected soon after flowering of the plant constitute the drug (Jain 1996). All parts of the plant are medicinal, but the leaves and seeds are the parts usually employed. Seeds are toxic, leaves sedative, narcotic, anodyne, antiseptic, also used in asthma and whooping cough. It is useful in relieving certain painful spasmodic condition of muscles. It also dilates eye pupils (Jain 1996; Singh and Panda 2005). Seeds are usually used as a paste and applied locally on pains (Jain 1996; Kaul 1997) also used in indigenous medicine, along with other ingredients for diabetes (Kaul 1997). With the advent of tissue culture it is now possible to produce sufficient plant material in a variety of species in a limited period of time and space, irrespective of the climatic conditions. So an attempt was made to develop a protocol for plantlet regeneration from *in vitro* shoot tips of *H. niger* using different types of cytokinins and to root them using several auxins.

MATERIALS AND METHODS

Certified seeds of *H. niger* were collected from the Regional Research Institute of Unani Medicines (RRIUM), University of Kashmir, and thoroughly washed with detergent (0.5% Cedepol) and 2-4 drops of Tween 20 (surfactant), then under running tap water followed by a final rinse with double distilled water. Surface sterilization of seeds was achieved by using 0.2% $HgCl_2$ for 20 min and a finally rinse 3–4 times with autoclaved double distilled water to remove all traces of sterilants.

Culture media

The basal medium consisted of Murashige and Skoog (1962) mineral salts and organic nutrients, 3% sucrose (Qualigens, India), 0.8% Difcobacto agar and different concentrations of plant growth regulators (PGRs; Himedia, Mumbai, India). All medium constituents were added and the pH was adjusted to 5.4 with 1 N NaOH or 1 N HCl and finally dispensed into 100-ml Erlenmyer flasks (borosilicate glass) plugged with non-absorbent cotton prior to autoclaving at 121°C and 15 psi for 20 min.

Experimental design

Seeds germinated and seedlings formed normally after 8 weeks of culture. From these seedlings shoot tips were excised and cultured on MS medium supplemented with 2.5, 5, 7.5, 10, 12.5, 15, 17.5 or 20 µM of 6-benzyladenine (BA), kinetin (Kn) and thidiazuron (TDZ), individually.

Cultures were maintained in an incubation room at 25 ± 3°C under a 16-hr photoperiod provided by cool white fluorescent tubes (3000 lux = 42 µmol/m^2/sec). The experiment was carried out in a completely randomized block design (CRD), repeated three times; each treatment had 10 replicates.

Received: 2 April, 2010. Accepted: 10 September, 2011.

Research Note

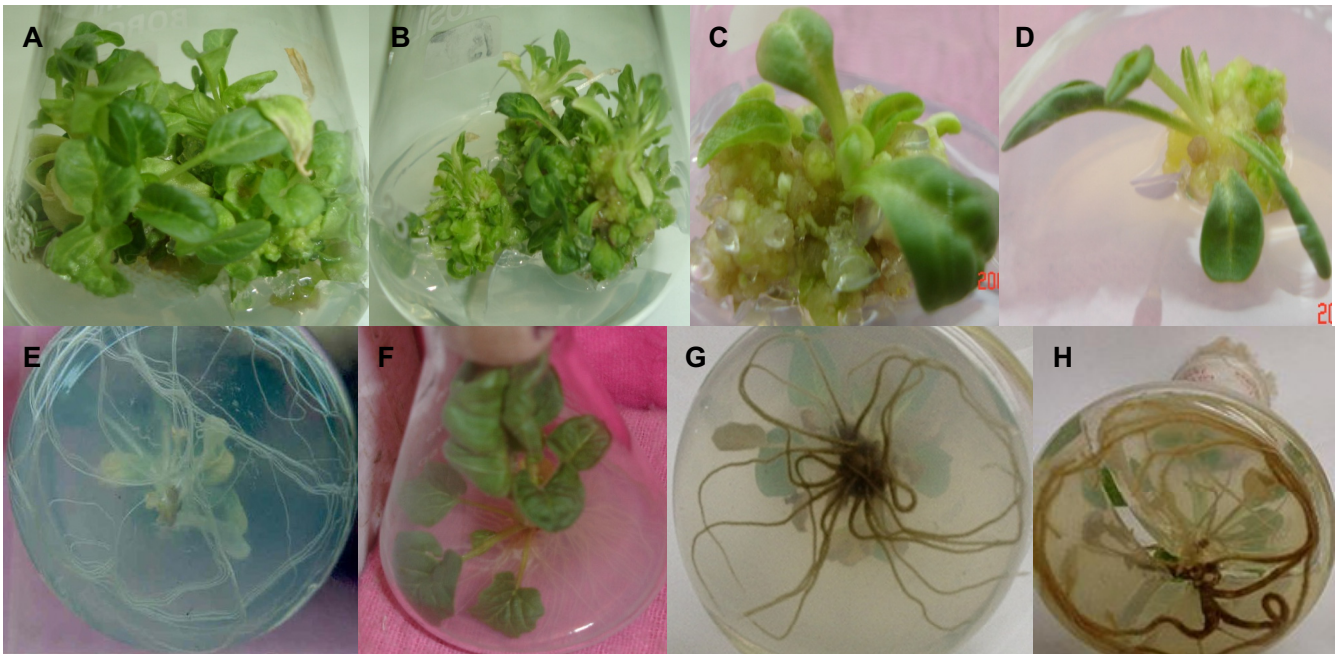

Fig. 1 ***In vitro*** **response of shoot tips of** ***Hyoscyamus niger*** **L.** (**A**) Increased shoot multiplication on MS + BA (12.5 µM). (**B**) Decreased shoot multiplication on MS + BA (20 µM). (**C**) Intense friable light greenish callus formation on MS+ TDZ (10 µM). (**D**) Moderate callus formation on MS + TDZ (20 µM). (**E**) Thin, long thread like multiple roots on MS medium. (**F**) Thick, long multiple roots on MS + IBA (4 µM). (**G**) Thick, long multiple roots on MS + NAA (2.5 µM). (**H**) Very thick and long multiple roots on MS + NAA (4 µM).

Table 1 Effect of MS medium with BA, Kn and TDZ on multiple shoot regeneration from *in vitro* shoot tips of *Hyoscyamus niger* L.

Concentration of PGRs used (µM)	BA Mean ± S.D.*	Kn	TDZ
2.5	9 ± 0.5	No response	Low callus
5	12 ± 0.2	-	Moderate callus
7.5	16 ± 0.5	-	Moderate callus
10	20 ± 0.4	-	Intense callus
12.5	27 ± 0.0	-	Moderate callus
15	22 ± 0.2	-	Moderate callus
17.5	20 ± 0.3	-	Moderate callus
20	17 ± 0.6	-	Moderate callus

*Data scored after 12 weeks of culture period; 10 replicates/treatment

Acclimatization

Rooted plantlets were removed from flasks and transferred in small pots containing sterile soil, sand, peat and vermiculite (1: 1: 1: 1, v/v).

Statistical analyses

No statistical analyses were applied because no response was recorded with Kn, only callus formation with TDZ, while the only response in terms of shoot number was recorded with BA so it was not possible to compare callus and shoot number between treatments.

RESULTS AND DISCUSSION

The main objective of this study was to develop an efficient and reproducible protocol for the micropropagation of *H. niger* through shoot tip culture. Full-strength MS medium supplemented with BA, Kn and TDZ at different concentrations were used in the present study (**Table 1**). Amongst the PGRs used, BA showed the best response. Shoots formed indirectly from callus at all concentrations of BA; the callus was nodular, compact and green and the whole callus clump differentiated into multiple shoots. The number of shoots increased up to 12.5 µM BA (27 multiple shoots per callus clump) (**Fig. 1A**). Higher concentrations of BA resulted in lower shoot multiplication (**Fig. 1B**). These findings were similar to earlier studies of Grewal *et al*. (1979) in shoot tips of *H. muticus*, Benjamin *et al*. (1987) in shoot tips of *Atropa belladonna*, Whipkey *et al*. (1992) in nodal segments of *Artemisia annua*, Zarate *et al*. (1997) in nodal explants of *A. baetica*, and Martin (2003) in shoot tips of *Rotula aquatica*. In all these studies shoot number decreased as BA concentration increased. These reports were further strongly supported by Sen and Sharma (1991); Rani and Groover (1999); Ray and Jha (2001) in *Withania somnifera* where again shoot number decreased with increasing BA concentration. No morphogenetic response was noticed with Kin which differs from the studies of Sen and Sharma (1991) and Ray and Jha (2001) in *W. somnifera*, Umer Sharief and Jagadish Chandra (1991) and Usha and Swamy (1994) in *Artemisia pallens*, and Martin (2003) in *R. aquatica* who reported shoot multiplication when Kn was used alone. When TDZ was used at similar concentrations as BA it promoted unorganized friable light-green callus, which did not differentiate. Callus formed up to 10 µM (**Fig. 1C**); a further increase in concentration decreased the amount of callus formed (**Fig. 1D**). Prathanturarug *et al*. (2003) in *Curcuma longa*, and Liu *et al*. (2003) in etiolated hypocotyls and seedlings of *Artemisia judaica* reported multiple shoot regeneration when TDZ was used. However, in this study, of all the cytokinins tested (BA, Kn and TDZ) best results were scored with BAP as compared to TDZ and Kn. However, in present study BA was found to be the superior PGR compared to TDZ and Kn with respect to shoot number. Different PGRs belonging to the same class may elicit a different morphogenetic response in a given tissue (Bhan 1998) as found for BA, Kn and TDZ in this study. Most PGRs are rapidly metabolized into physiologically inactive compounds (Bhan 1998), which might explain why Kn did not show any response.

Multiple shoots elongated and formed thin, long, thread-like adventitious roots after 4 and 8 weeks of culture (**Fig. 1E**). The rooting potential of IBA, IAA and NAA at 0.5-5.0 µM was assessed (**Table 2**) and scored after 12 weeks of culture on the same medium. Different concentrations of IBA resulted in multiple root formation, maximum at 4 µM (**Fig. 1F**; 12.5 ± 0.5 roots/shoot). Higher IBA concentrations decreased root number. Muthukumar *et al*. (1998, 2000, 2001, 2003) in *Datura metel* and Britto *et al*. (2002) in *Solanum incanum* also reported rhizogenesis at different concentrations of IBA.

No rooting response was noticed with IAA while with NAA, best rooting response was achieved on 2.5 µM NAA,

Table 2 Effect of MS medium with different concentrations of IBA, IAA or NAA on root regeneration of *Hyoscyamus niger* L.

IBA (µM)	IAA (µM)	NAA (µM)	Root No. (Mean ± S.D.)*	Percentage response
0.5	-	-	4.0 ± 0.2	100
1.0	-	-	5.0 ± 0.2	100
1.5	-	-	6.0 ± 0.2	100
2.0	-	-	8.0 ± 0.2	100
2.5	-	-	9.0 ± 0.5	100
3.0	-	-	9.0 ± 0.5	100
3.5	-	-	9.5 ± 0.5	100
4.0	-	-	12.5 ± 0.5	100
4.5	-	-	10.0 ± 0.5	100
5.0	-	-	9.0 ± 0.2	100
-	0.5	-	-	-
-	1.0	-	-	-
-	1.5	-	-	-
-	2.0	-	-	-
-	2.5	-	-	-
	3.0	-	-	-
	3.5	-	-	-
	4.0	-	-	-
	4.5	-	-	-
	5.0	-	-	-
	-	0.5	5.0 ± 0.2	100
	-	1.0	5.5 ± 0.2	100
	-	1.5	10.5 ± 0.5	100
	-	2.0	12.5 ± 0.5	100
	-	2.5	14.5 ± 0.5	100
	-	3.0	10.0 ± 0.5	100
	-	3.5	8.0 ± 0.5	100
	-	4.0	5.5 ± 0.5	100
	-	4.5	3.5 ± 0.5	100
	-	5.0	6.0 ± 0.5	100

Data scored after 12 weeks of culture period; 10 replicates/treatment

with 14.5 ± 0.5 roots/shoot (**Fig. 1G**). Roots at 4 µM NAA were thick but few (5.5 ± 0.5 roots/shoot; **Fig. 1H**). However, at 5 µM NAA roots were similar to those obtained on MS basal medium (i.e., the control). Eapen *et al*. (1978) in *Atropa belladonna*, Lorz *et al*. (1979) in *Hyoscyamus albus* and *H. muticus*, Banerjee *et al*. (1985) in *Solanum sarrachoides*, Arora and Bhojwani (1989) in *Saussurea lappa*, Zarate *et al*. (1997) in *Atropa baetica*, and Casado *et al*. (2002) in *Santolina canscens* noted root regeneration with NAA.

Rooted plantlets only showed 50% survival rate since plants were acclimatized outside under natural conditions and since winter was approaching.

ACKNOWLEDGEMENTS

The authors are highly thankful to Director CORD for providing the necessary laboratory facilities for the work undertaken.

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