Regeneration of Multiple Shoots from Immature Seeds and Mass Multiplication of Dhatura (*Datura metel* L.)

A. Yadav, N. Joshee and A.K. Yadav
Agricultural Research Station
Fort Valley State University
1005 State University Drive
Fort Valley, GA 31030
USA

Keywords: multiple shoot regeneration, liquid lab rocker bioreactor, biomass production

Abstract

Dhatura (*Datura metel* L., Solanaceae) also called 'Thorn Apple' or 'Indian Apple' is widely found in India, Pakistan, Africa, Mediterranean region, Southern China, USA and Europe. It is often included in the list for poisonous plants because it contains toxic tropane alkaloids, which are mainly responsible for its medicinal properties. The dhatura plant contains such alkaloids hyoscyamine (atropine) and hyoscine generally in 2:1 ratio while the total alkaloid content ranges between 0.25% and 0.5%. Dhatura is an annual plant growing up to 4 ft tall, and is susceptible to frost since it is hardy to zone 9 only. It flowers from June to July. The plant prefers well-drained sandy loam soil. It flourishes in the neutral and basic soils but can grow in very alkaline soil as well. Increasing global demand for herbal medicines makes it inevitable to mass multiply this plant for the year round availability by utilizing biotechnological tools, which will not only ensure the mass production of clean plant material but will also help in the conservation of this species. Therefore, we have started biotechnological investigations and have developed in vitro plant regeneration protocols utilizing fortification of media with different combinations of plant bioregulators. It has been observed that the thin cell layer (TCL) culture medium supplemented with 2,4-D (2.25 mg/L) and BAP (0.25 mg/L) was found suitable to induce morphogenic callus in immature seed explants while the MS medium supplemented with BAP (1.0 mg/L) was found to be optimum for multiple shoot regeneration. Liquid Lab Rocker Bioreactor system utilizing liquid MS medium supplemented with BAP produced significantly greater biomass of dhatura than did the magenta boxes containing the semi-solid MS medium supplemented with BAP.

INTRODUCTION

Dhatura (*Datura metel* L., Solanaceae) which is also called 'Thorn Apple' or 'Indian Apple' is widely found in India, Pakistan, Africa, Mediterranean region, Southern China, USA and Europe. Dhatura is cultivated in the USA and Europe, and occurs as a weed all over the world (Daniel, 2006). Tropane alkaloids are mainly responsible for its medicinal properties; these alkaloids are toxic in nature and because of that dhatura species are listed among the poisonous plants. It is an annual plant, growing 3–6 feet in height with branching stem and alternate slightly pinnatified leaves. It blooms during summer months and large flowers are solitary in leaf axils. The scented flowers are hermaphrodite and are pollinated by Insects. The quadrilocular erect capsule is very prickly and contains numerous brown seeds. It is hardy to zone 9 in the USA and is quite frost susceptible. It cannot grow in the shade. The plant prefers well-drained sandy loam soil. It thrives well in the neutral and alkaline soils but can grow in very alkaline soil as well.

Bioactive Compounds

The total alkaloid content ranges between 0.25% and 0.5%, of which the major alkaloids, hyoscyamine (atropine) and hyoscine occur in 2:1 ratio. Higher levels of

hyoscine have been reported from young shoots, however. Seeds yield about 0.2% alkaloids and 15–30% fixed oil. The dhatura root contains ditigloyl esters and some alkamines along with hyoscyamine and hyoscine (Daniel, 2006). Since atropine accomplishes dilation of the pupils it is thus used in eye surgery.

Traditional/ Medicinal Properties

The whole plant and specially the leaves and seeds, possess antiasthmatic, anaesthetic, anodyne, antitussive, antispasmodic, bronchodilator, hallucinogenic, hypnotic, and mydriatic properties (Emboden, 1979; Duke and Ayensu, 1985; Muller, 1998; Weitz, 2003; Ertekin et al., 2005). In India, it has a wide range of applications, including for treating epilepsy, hysteria, insanity, heart diseases, fever with catarrh, diarrhea, skin diseases, etc. (Emboden, 1979; Chopra et al., 1986). A poultice of crushed leaves is used to relieve pain (Nguyen and Doan, 1989). In China, the whole plant is used for treating Asthma (Duke and Ayensu, 1985). In Vietnam, the dried flowers and leaves are cut into small chips and used in antiasthmatic cigarettes (Nguyen and Doan, 1989). The occurrence of anti-cholinergic alkaloids, tropane alkaloids, in dhatura plant contributes to its antiasthmatic properties (Friedman, 2004). The toxic dose is very close to the medicinal dose so this plant should only be used under the guidance of a qualified practitioner.

The dhatura plant biomass for bioactive phytochemicals and herbal medicines is mostly collected from the wild resources. Increasing global demand makes it inevitable to mass multiply this plant for the year round availability by utilizing the biotechnological tools, which will not only ensure the mass production of clean plant material but will also help in the plant conservation of this species. There are several reports available on the plant regeneration of dhatura propagating through various tissues; however, no report is available on multiple shoot regeneration through callusing induced from seeds. The present study was undertaken to determine the suitable seed stage to produce morphogenic callus, selection of media, and standardization of plant bioregulator(s) for callusing, multiple shoot regeneration, and maximum biomass production of dhatura.

MATERIALS AND METHODS

Explant Collection and Sterilization

The fresh dhatura fruits at three different stages of maturity (Fig. 1a) were collected from the *Datura metel* plants growing wild and were kept under running tap water for 1 h and then sterilized with 10% clorox solution for 15 min. After the clorox treatment, fruits were washed four times with double distilled autoclaved water. The sterilized fruits were cut in to two halves (Fig. 1b) with the scalpel and immature seed explants from all three different stages (Seed Stage I, Seed Stage II and Seed Stage III) as presented in Fig. 1c, were collected in different petridishes containing double distilled autoclaved water and 1% plant preservative mixture (PPM) solution (Plant Cell Technology, Inc., Washington DC). The immature seeds from all three stages of fruits were soaked in 1% PPM for 1 h before culturing in to different media combinations for callus induction.

Culture Medium and Conditions

Modified TCL (thin cell layer culture medium, Van and Le, 2000) supplemented with 6% sucrose (w/v) and MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose (w/v), solidified with 2.5 g/L Phytagel (Sigma-Aldrich Inc., USA), and various concentrations of plant bioregulators (2,4-D and IAA auxins and BAP and Kin cytokinins) in different combinations were used for in vitro experiments. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCl before autoclaving at 121ºC for 22 minutes. Cultures were incubated at 25 ±1°C either in dark for callus induction or under cool white florescent light (40 µmol m^{-2} s^{-1}) for multiple shoot regeneration and biomass production with 16 h photoperiod. Subculturing in to fresh media for multiple

shoot regeneration and biomass productions was done at 15 days interval.

TREATMENTS

Morphogenic Callus Induction

For morphogenic callus induction, each treatment was replicated thrice and 10 sterilized immature seeds were cultured per replication (i.e., in each petri dish) for all three stages of dhatura fruits and were kept in dark at room temperature.

T_1 = MS (Control)
T_2 = MS + 2, 4-D (0.5 mg/L) + BAP (0.25 mg/L)
T_3 = MS + 2, 4-D (1.25 mg/L) + BAP (0.25 mg/L)
T_4 = MS + 2, 4-D (2.25 mg/L) + BAP (0.25 mg/L)
T_5 = MS + IAA (0.5 mg/L) + BAP (0.25 mg/L)
T_6 = MS + IAA (1.25 mg/L) + BAP (0.25 mg/L)
T_7 = MS + IAA (2.25 mg/L) + BAP (0.25 mg/L)
T_8 = TCL (Control)
T_9 = TCL + 2, 4-D (0.5 mg/L) + BAP (0.25 mg/L)
T_{10} = TCL + 2, 4-D (1.25 mg/L) + BAP (0.25 mg/L)
T_{11} = TCL + 2, 4-D (2.25 mg/L) + BAP (0.25 mg/L)
T_{12} = TCL + IAA (0.5 mg/L) + BAP (0.25 mg/L)
T_{13} = TCL + IAA (1.25 mg/L) + BAP (0.25 mg/L)
T_{14} = TCL + IAA (2.25 mg/L) + BAP (0.25 mg/L)

Where, MS = Murashige and Skoog (1962) culture medium
TCL = Modified thin cell layer culture medium (Table 4)
2, 4-D = 2,4-Dichlorophenoxy acetic acid
BAP = 6-benzylaminopurine
IAA = Indole-3-acetic acid

Multiple Shoot Regeneration

The morphogenic callus lumps obtained from Seed Stage II were further subcultured on to MS medium for two weeks and then transferred to the MS medium supplemented with BAP or Kinetin. For the multiple shoot regeneration from morphogenic callus induced from immature seeds of dhatura fruit Stage II, each treatment was replicated three times and 5 callus-induced immature seeds were cultured in each petridish per replication and were kept under the light condition with 16 h photoperiod.

T_1 = MS (Control)
T_2 = MS + BAP (0.5 mg/L)
T_3 = MS + BAP (1.0 mg/L)
T_4 = MS + BAP (1.5 mg/L)
T_5 = MS + BAP (2.0 mg/L)
T_6 = MS + Kin (0.5 mg/L)
T_7 = MS + Kin (1.0 mg/L)
T_8 = MS + Kin (1.5 mg/L)
T_9 = MS + Kin (2.0 mg/L)
T_{10} = MS + BAP (0.5 mg/L) + Kin (0.5 mg/L)
T_{11} = MS + BAP (1.0 mg/L) + Kin (1.0 mg/L)
T_{12} = MS + BAP (1.5 mg/L) + Kin (1.5 mg/L)
T_{13} = MS + BAP (2.0 mg/L) + Kin (2.0 mg/L)

Where, MS = Murashige and Skoog medium
BAP = 6-benzylaminopurine
Kin = Kinetin (N6-furfuryladenine)

Plant Biomass Production

For the biomass production dhatura plants, eight treatments were used with each treatment replicated four times. After weighing on UV sterilized balance in the hood, 10 g of fresh weight explants (multiple shoot induced) were cultured in each replication per treatment.

T_1 M_1 D
T_1 M_2 D
T_2 M_1 D
T_2 M_2 D
T_3 M_1 D
T_3 M_2 D
T_4 M_1 D
T_4 M_2 D

Where, T_1 = MS (Control)
T_2 = MS + BAP (0.5 mg/L)
T_3 = MS + BAP (1.0 mg/L)
T_4 = MS + BAP (2.0 mg/L)
M_1 = Semi Solid MS Media in Magenta Boxes
M_2 = Liquid MS Media in Liquid Lab Rocker Bioreactors
D = Dhatura Biomass Increment after No. of Days Subculturing

OBSERVATIONS

Observations were recorded for the morphogenic callus induction in immature seeds, number of multiple shoots regenerated per immature seeds, and biomass increment by weighing the explant with the Magenta Box or Liquid Lab Rocker Bioreactors on balance at every 15 days up to 60 days after culturing in to Semi Solid or Liquid MS Media. Increase in fresh biomass was calculated by subtracting the weight of without explant (only media) Magenta Box or Liquid Lab Rocker Bioreactors from the total weight of the explant and media contained in the Magenta Boxes or Liquid Lab Rocker Bioreactors.

Acclimatization and Hardening of Plants

After the development of root system, the plantlets were carefully removed from the medium. The root system was washed thoroughly under gentle running tap water to remove any traces of agar adhering to the plantlet. The cleaned plantlets were planted in eight inch plastic pots containing potting mixture (Pro-Mix "BX", Cassca, Montgomery, AL). The pots were kept in the mist chamber for acclimatization and after one week shifted to the greenhouse for hardening. After the acclimatization and hardening for one month, the plants were transferred to the field.

Statistical Analysis

All the experiments were carried out in a completely randomized design (Snedecor and Cochran, 1987). Significance was determined by analysis of variance (ANOVA) and critical differences (CD) between treatment means at p α= 0.05.

RESULTS AND DISCUSSION

Morphogenic Callus Induction

It has been noted that the only modified thin cell layer (TCL) culture medium supplemented with 2,4-D (2.25 mg/L), BAP (0.25 mg/L) and sucrose 6% (w/v) was found suitable to induce morphogenic callus in immature seed explants. The immature Seed Stage II only showed morphogenic callusing after three weeks of subculturing (Table 1,

Fig. 1e). There was no callusing observed in either of the controls (MS and TCL). Most of the treatment combinations induced callusing in Seed Stages I and II; however, in Seed Stage III, the higher levels of auxins were able to induce callusing, but there was no morphogenic response in either of Seed Stage I and III (Table 1). In the present study, the different combinations of hormones yielded different types of calli may be due to the requirement of different bioregulators for the initiation of morphogenic callus varies due to the endogenous levels of hormones in the explants. Hooker and Nabors (1977) recorded an interaction between the type and concentrations of plant bioregulators, and explant source, a combination of 2,4-D and BAP resulting in a higher callus production than the combinations of IAA or NAA with Kinetin. Krens and Jamar (1989) also reported that auxins were more effective in inducing callusing when combined with low BAP levels.

Multiple Shoot Regeneration

The morphogenic callus lumps produced from Seed Stage II were further subcultured on to the MS medium for two weeks and then transferred to the MS medium supplemented with BAP or Kinetin and sucrose 3% (w/v). Multiple shoot primordia initiation was observed after two weeks of subculturing (Fig. 2g and h). MS medium supplemented with BAP (1.0 mg/L) produced maximum number of multiple shoots per seed and it was significantly higher than all other treatments (Table 2). There was no shoot regeneration at all observed in the control. MS media supplemented with BAP alone produced more number of multiple shoots than Kinetin or the BAP-Kinetin combinations (Table 2). Similar response of BAP was also reported by Ulcer and Mollamehmetoglu (2001) in Linden (*Tilia platyphyllos* Scop); Gurel et al. (2001) in sugar beet, and Yadav et al. (2006) in brahmi.

Plant Biomass Production

For dhatura plant biomass production, 10 g fresh multiple shoot induced explant was weighed in hood on UV sterilized balance and cultured in to semi solid MS medium (SSM) or liquid MS medium (LM) supplemented with different levels of BAP and 3% (w/v) sucrose. Biomass increment was recorded at the 15 days interval after subculturing. Results revealed that there was significant effect of increasing the levels of BAP on the dhatura biomass production in both SSM in Magenta Boxes and LM in LLR Bioreactors (Table 3). Dhatura plantlets cultured in LM in LLR Bioreactors produced significantly higher biomass than the plantlets cultured on to SSM in Magenta Boxes. Significant increase in dhatura plant biomass was observed at every 15 days interval up to 60 days of subculturing in all the treatment combinations (Table 3). Large number of multiple shoots produced from the nodes of the plantlets transferred to the LLR Bioreactors (Fig. 2j). Interactions between all the treatment combinations were also significant. The continuous gaseous exchange and movement of media in bioreactors helps in minimizing the toxic substances in media which lead to higher biomass production. The cultivation in liquid media was also reported to improve plant quality and multiplication rates of banana, coffee, and rubber (Alvard et al., 1993; Teisson and Alvard, 1995; Etienne et al., 1997).

Rooting of Plants

Dhatura plants produced profuse roots with or without additions of IBA (1.0 mg/L) in either SSM or LM. Rooted dhatura plants were transferred to the greenhouse for acclimatization and then field transfer.

CONCLUSIONS

The system established in the present study for callus induction using immature seeds followed by multiple shoot regeneration could be used for introducing gene(s) of interest in dhatura with necessary modifications.

ACKNOWLEDGEMENTS

The authors are thankful to the Agricultural Research Station of the Fort Valley State University, Fort Valley, Georgia, for providing laboratory and other research facilities to carryout the present investigation.

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Tables

Table 1. Morphogenic callus induction in different seed stages of dhatura.

Treatments	Media and Plant Growth Regulators	Dhatura seed stages		
		Stage I	Stage II	Stage III
T_1	MS (Control)	-	-	-
T_2	MS + 2,4-D (0.5 mg/L) + BAP (0.25 mg/L)	+	-	-
T_3	MS + 2,4-D (1.25 mg/L) + BAP (0.25 mg/L)	+	+	-
T_4	MS + 2,4-D (2.25 mg/L) + BAP (0.25 mg/L)	+	+	+
T_5	MS + IAA (0.5 mg/L) + BAP (0.25 mg/L)	-	-	-
T_6	MS + IAA (1.25 mg/L) + BAP (0.25 mg/L)	+	+	-
T_7	MS + IAA (2.25 mg/L) + BAP (0.25 mg/L)	+	+	+
T_8	TCL (Control)	-	-	-
T_9	TCL + 2,4-D (0.5 mg/L) + BAP (0.25 mg/L)	+	+	-
T_{10}	TCL + 2,4-D (1.25 mg/L) + BAP (0.25 mg/L)	+	+	-
T_{11}	TCL + 2,4-D (2.25 mg/L) + BAP (0.25 mg/L)	+	++	+
T_{12}	TCL + IAA (0.5 mg/L) + BAP (0.25 mg/L)	+	-	-
T_{13}	TCL + IAA (1.25 mg/L) + BAP (0.25 mg/L)	+	+	-
T_{14}	TCL + IAA (2.25 mg/L) + BAP (0.25 mg/L)	+	+	+

Note: - No Callusing, + Callusing, ++ Morphogenic Callusing.

Table 2. Multiple shoot regeneration from morphogenic calluses in dhatura seeds Stage II.

Treatments	No. of multiple shoots regenerated/seed
T_1 MS (Control)	0.00
T_2 MS + BAP (0.5 mg/L)	4.40
T_3 MS + BAP (1.0 mg/L)	11.06
T_4 MS + BAP (1.5 mg/L)	9.86
T_5 MS + BAP (2.0 mg/L)	9.40
T_6 MS + Kin (0.5 mg/L)	2.73
T_7 MS + Kin (1.0 mg/L)	7.80
T_8 MS + Kin (1.5 mg/L)	8.93
T_9 MS + Kin (2.0 mg/L)	9.00
T_{10} MS + BAP (0.5 mg/L) + Kin (0.5 mg/L)	8.00
T_{11} MS + BAP (1.0 mg/L) + Kin (1.0 mg/L)	8.93
T_{12} MS + BAP (1.5 mg/L) + Kin (1.5 mg/L)	8.80
T_{13} MS + BAP (2.0 mg/L) + Kin (2.0 mg/L)	9.00
p α= 0.05	0.92
CV	7.35

Table 3. Effect of BAP concentrations and type of media on dhatura plant biomass production.

Parameters	Dhatura plant biomass production (g/culture vessel)										
	Semi solid media (M_1) (Magenta boxes)				Mean	Liquid media (M_2) (LLR bioreactors)				Mean	Over all mean
	15 D	30 D	45 D	60 D		15 D	30 D	45 D	60 D		
MS (Control) (T_1)	16.23	24.58	39.94	51.90	33.16	18.42	28.10	46.39	60.97	38.47	35.81
MS + BAP (0.5 mg/L) (T_2)	20.32	28.46	42.55	57.13	37.11	24.85	33.41	54.41	64.24	44.23	40.67
MS + BAP (1.0 mg/L) (T_3)	26.61	37.44	52.40	68.08	46.13	34.45	46.47	68.00	79.43	57.09	51.61
MS + BAP (2.0 mg/L) (T_4)	29.30	40.87	55.63	70.71	49.13	36.63	49.71	70.74	82.13	59.80	54.46
Mean	23.12	32.84	47.63	61.95		28.58	39.42	59.88	71.69		
Over all mean	41.38					49.89					
Interactions	p α= 0.05	SE ±									
Hormone levels (T)	0.79	0.34									
Media type (M)	1.12	0.48									
Biomass increment No. of days after subculturing (D)	1.12	0.48									
T*M	1.59	0.68									
T*D	1.59	0.68									
M*D	2.25	0.96									
T*M*D	3.18	1.37									
CV %	6.00										

Table 4. Modified TCL culture medium composition.

Macro elements {TCL Culture Medium (Van and Le, 2000)}	
KNO_3	2214 mg/L
$CaCl_2.2H_2O$	184 mg/L
$MgSO_4.7H_2O$	303 mg/L
$NH_4H_2PO_4$	300 mg/L
Micro elements {Murashige and Skoog (1962) Medium}	
H_3BO_3	6.2 mg/L
$CoCl_2.6H_2O$	0.025 mg/L
$CuSO_4.5H_20$	0.025 mg/L
Na_2EDTA	37.3 mg/L
Fe $(SO_4).7H_2O$	27.8 mg/L
$MnSO_4.H_2O$	16.9 mg/L
KI	0.83 mg/L
$Na_2MoO_4.2H_2O$	0.25 mg/L
$ZnSO_4.7H_2O$	8.6 mg/L
Vitamins {Modified Murashige and Skoog (1962) Medium}	
Thiamine HCl	100 mg/L
Glycine	2.0 mg/L
Nicotinic acid	0.5 mg/L
Pyridoxine HCl	0.5 mg/L
myo-Inositol	100 mg/L
Carbohydrate	
Sucrose	60 g/L
Amino acids	
Glutamine	400 mg/L
Casein Hydrolysate	100 mg/L

Figures

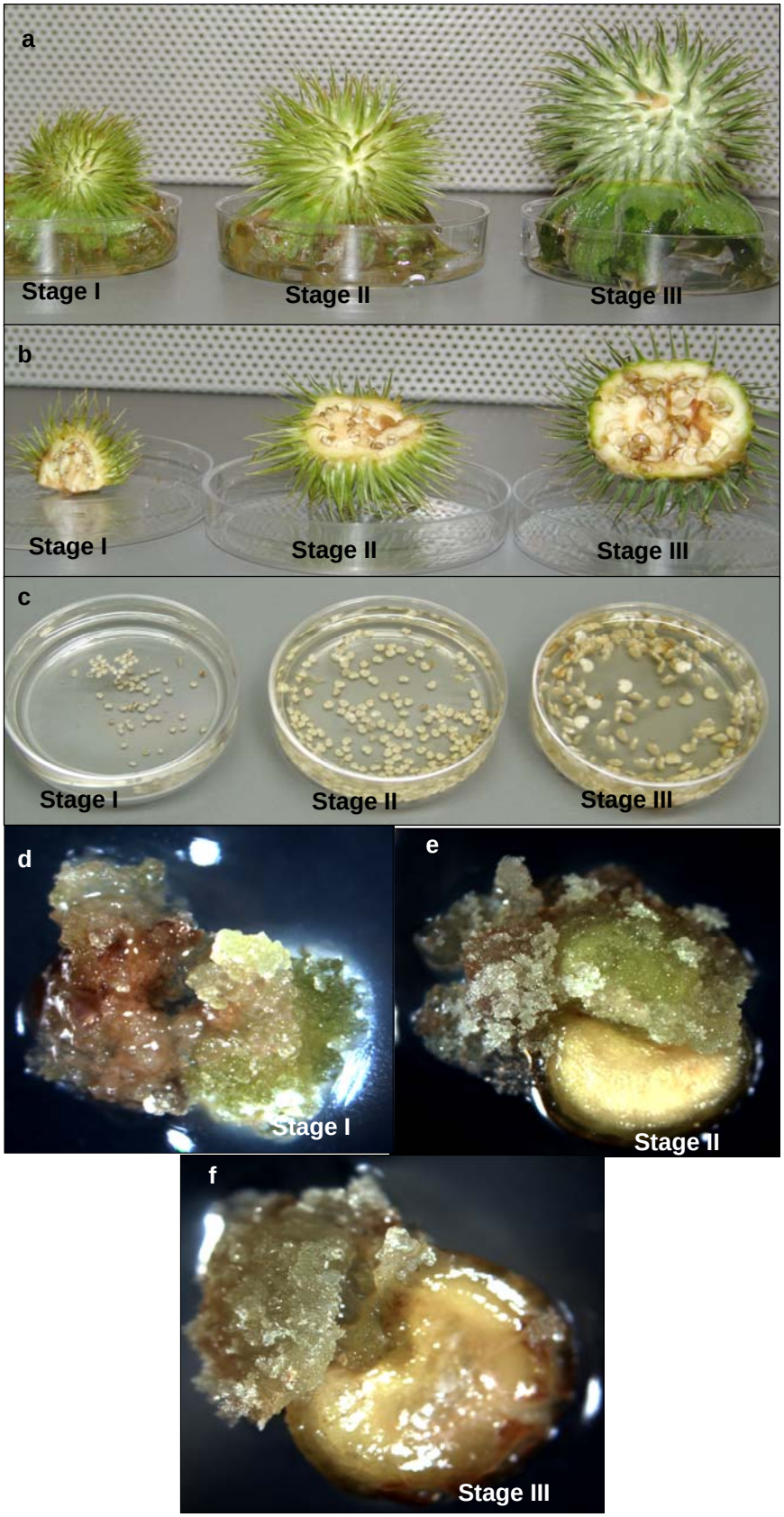

Fig. 1. Explant selection and morphogenic callus induction in immature seeds of dhatura. (a) Different stages of dhatura fruits, (b) T.S. of dhatura fruits, (c) Immature dhatura seeds, (d-f) Callus induction in immature dhatura seeds.

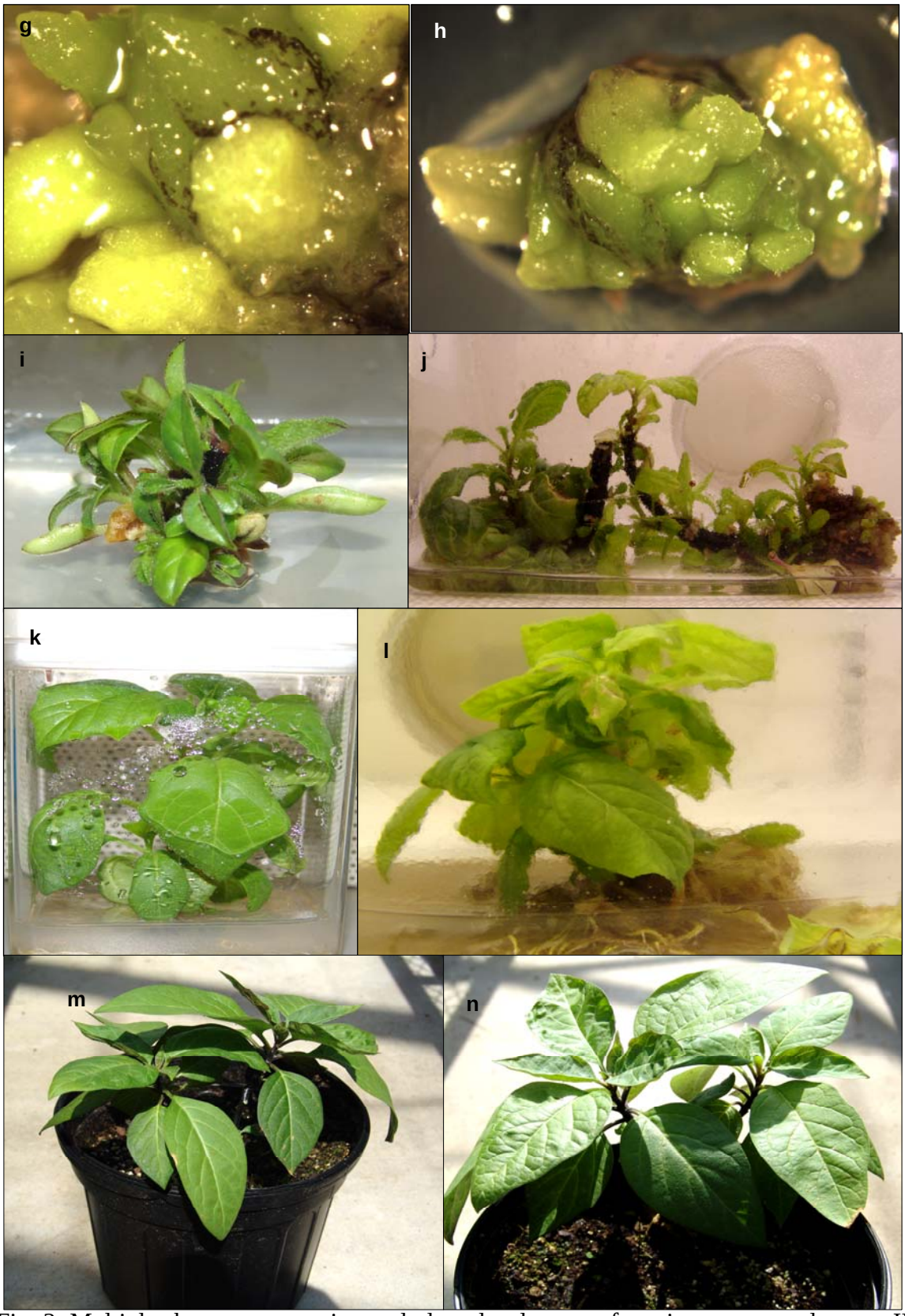

Fig. 2. Multiple shoots regeneration and plant development from immature seeds stage II of dhatura, (g-h) Multiple shoot primordia initiation in dhatura seed stage II, (i) Multiple shoot regeneration in SSM in magenta box, (j) Multiple shoot regeneration in LM in LLR bioreactor, (k) Dhatura plant growing in SSM in magenta box, (l) Dhatura plant growing in LM in LLR bioreactor, (m) Acclimatized dhatura plant in greenhouse.