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In Vitro Callus Induction and Regeneration of Medicinal Plant *Datura innoxia*

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Abstract: The present study indicate the possibility of the development of callus from stem cut, leaf and root explants and regeneration of Datura plant (*Datura innoxia*) on Murashige and Skoog (MS) media supplemented with different concentrations of 6-Benzylaminopurine (BA) and 1-Naphthaleneacetic (NAA). Sterilized explants were inoculated and incubated in culture shade under different conditions (light and dark). Data analysis showed that maximum percentage (83%) of calli were induced from stem cut explants on MS media supplemented with NAA and BA at 0.5 and 1.0 mg/l respectively under dark condition. Maximum fresh and dry weight was found 395.63 mg and 35.64 mg respectively with supplementation of 0.5 mg/l NAA and 1 mg/l BA. Stem cut derived calli were transfer to MS regeneration medium supplementing different concentrations of plant regulators (PGRs) under different conditions. Maximum regeneration (78%) was found on MS media supplementing 1 mg/l of BA and 1 mg/l off NAA under dark condition. The attempt for callus induction from the explants (stem, leaf, root) using MS media with supplementation of different combinations of 2, 4-Dichlorophenoxyacetic acid (2, 4-D), BA and NAA, Kinetin (kin) under both light and dark conditions were carried out but no significant results were found.

Keywords: *Datura innoxia*, Callus, Regeneration, 1-Naphthaleneacetic, 6-Benzylaminopurine

Introduction

Datura plant belongs to the Solanaceae family and includes *Datura* spp, *Datura innoxia*, Datura metal, Datura stramonium, it is a seasonal herb plant with a height of 60-100cm, a smooth cylindrical stem and smelly. Leaves are simple, oval, serrated, spruce, green and have a distinctive smell and flowers are large single trumpet white. The plant starts flowering from June and yields in July (Siddiqui *et al*., 1988). Datura plant exists in all regions of the world except frozen areas; it grows wild in all warm and temperate regions of the world and is widely distributed in tropical and temperate regions (Ulrike *et al*., 2002).

Phytochemical investigation on Datura revealed that methanolic and hydroalcoholic extracts of dried seeds, leaves and flowers contain alkaloids, tannins, cardiac glycosides, flavonoids, carbohydrates, amino acids and phenolic compounds (Ratan, 2011) Traditionally the dried leaves, flowers and roots of Datura plants were used as narcotic, antispasmodic, antitussive, bronchodilator, anti-asthmatic and as hallucinogenic. Datura plants was also used in diarrhea, skin diseases, epilepsy, hysteria, painful menstruation, skin ulcers, rheumatic pains, wounds and burns, hemorrhoids. In ayurveda, the plant was considered germicide, anodyne, antiseptic, bitter, acrid, astringent, antiphlogistic, narcotic and sedative (Parrotta, 2001; IMHFW, 2008). Datura is used as a hypnotic, analgesic and dilute agent, especially colic, intestine and spinal cord fracture. It has great pharmacological value and has antimicrobial, insecticidal, antiinflammatory, analgesic and antipyretic, antispasmodic and spasmogenic, neurological, antioxidant, cytotoxic, wound healing, antidiabetic, xanthine oxidase inhibitory activity and reproductive effects (Al-Snafi and Ali Esmail, 2017).

Datura plant is also highly toxic to humans and animals and its side effects include headaches, nausea, dizziness, extreme thirst and dryness, burning of the skin, loss of sight and movement and severe cases leading to death (Siddiqui *et al*., 1988).

Thus *Datura innoxia* demands a vast research for improvement and find the insights on this medicinally important plant. For further improvement, a defined tissue culture protocol for molecular researches is utmost necessary. Many biotechnological especially tissue culture approaches, have been successfully employed for in-vitro propagation of many plants. Plant regeneration from callus, somatic embryos or buds depended on genotype, explants source, media type (Hnana *et al*., 2004; Prodhan *et al*., 2016). Amiri *et al*. (2011) showed that initiation of callus from cultured leaf explants on MS media supplemented with 2mg/l 2,4-D+0.5 mg/l kin. Also, cultured the mature embryo of Datura in MS media equipped with 2 mg/l 2,4-D only lead to obtaining callus. Differentiation of the callus induction from the leaf explants to branches when cultured on MS media equipped with 3 mg/l BA+1 mg/l NAA, Also the callus induced from the embryo differentiation to vegetative branches when cultured in MS media containing 2mg/l BA+1 mg/l NAA. Root explants of Datura cultured in MS media containing 4 mg/l BA led to its differentiation into Somatic embryogenesis after 15 days from culture. When these embryos transferred to MS media containing 2 mg/l BA and 1 mg/l gibberellic acid (GA), they extended the vegetative branches (Amiri *et al*., 2011). The callus was induced from cotyledons of Datura when culturing in MS media contain 2 mg/l 2,4-D with 0.25 mg/l kin and 2 mg/l casein hydrolyzed, callus differentiation to vegetative branches when cultured in MS media containing 1 mg/l BA+2 mg/l NAA (Kinsara *et al*., 1994). Mawahib *et al*. (2014) noted that leaf of Datura cultured in MS Media Supplemented with 0.05 mg/l 2, 4-D and 0.025 mg/l kin given callus at a rapid rate compared to culture in MS media containing 2, 4-D alone. The tissue culture technology helps plant propagation throughout the year without restricting the cultivation season as well as reducing the distance required for cultivation (Hasan *et al*., 2016a). The aim of this study is to determine the appropriate explants for callus induction and callus derived shoot induction along with the best combinations of Plant Growth Regulators (PGRs).

Materials and Methods

Preparation of the Culture Media

Murashigr and Skoog (1962) media was prepared by dissolving organic and inorganic salts with supplementation of 3% sucrose, 0.7% agar and different concentrations of Plant Growth Regulators (PGRs). pH of the media was adjusted with 5.8-6.0. After completion of adding the components, media was melted in oven and 10 mL media poured into each test tube (2.5×8 cm) and sealed with cotton made cork. The media was sterilized at 121°C temperature, 15 psi pressure for 20 min (Hasan *et al*., 2019).

Sterilization of Explants

The explants (stems, leaves and roots) were separated from juvenile Datura, wash with running water several times for one hour and rinsed in 75% ethanol (v/v) for 30 seconds. Subsequently the explants were washed with sterilized distilled water for several times. Again explants were immerged in 1% sodium hypochlorite (NaOCl) for 3 min and then washed several times with sterile distilled water. Then the explants were dried using sterilized filter paper (Hasan *et al*., 2016b).

Callus Induction

For callus induction, sterilized explants were inoculated in the MS media supplemented different concentrations and combinations of plant growth regulators BA, 2.4-D, kin and NAA (Table 1). Inoculated explants were incubated in dark (50%) and light (50%) condition under 25±2°C temperature and intensity of light were 2000 lux.

Measurement of Fresh and Dry Weight

Fresh weights of inducted callus were measured after month of inoculation and this work was carried out under laminar air flow conditions. The dry weights of callus measured after the treatment at 72°C for 48 h.

Regeneration Media

The inducted callus were regenerated in MS media supplemented with different concentration of BA and NAA (Table 4). The inoculated explants for regeneration incubated in culture room under 25±2°C temperature and 2000 lux light intensity.

Statistical Analysis

The experiment was designed using Randomized Complete Block Design (RCBD) and the results were analyzed using a Duncan's test at a probability level of 0.05 (Glaser and Biggs, 2010).

Results

Selection of Explants and Culture Media for Callus Induction

All explants (stems, leaves and roots) has failed to induced callus when cultured in MS media supplemented with different combinations of growth regulators

(BA+2,4-D) and (kin+NAA) at concentration of (0, 0.5, 1, 1.5, 2 mg/l) for both 2,4-D and NAA; at concentration of (0, 0.5, 1, 1.5 mg/l) for both kin and BA (Sharma *et al*., 1993) in light and dark conditions, So these were excluded from subsequent experiments. When cultured the same explants in MS media supplementing NAA at concentration of (0, 0.5, 1, 1.5, 2 mg /l) and BA of (0, 0.5, 1, 1.5 mg /l) produced callus but root explants showed the negative effect and failed to induced callus in light and dark conditions, so root explants were excluded in subsequent experiments. The callus was induced when inoculated cultures with leaf explants in this media maintained dark conditions only. It took a long time to induced callus and for a few combinations and not for each concentration and callus needed to sub-culture at interval of 10-12 days because callus was turning brown, shrinking, dying (Fig. 1a). Stem cut explants under dark conditions led to the callus when cultured with same media, while the callus was not induced in light conditions. Stem cut induced calli were good for most concentrations and its type was friable (Fig. 1b). Therefore this combination and the explants were adopted for conducting this study. The callus induction from leaves was excluded because it failed to survive when the sub culture was delayed and its quantity was very low. Maybe the cause of the difference of calli induced from the explants of Datura due to the types and source of the explants, totopotency in plant cells and the difference in the number of cells able to divide in these parts and their internal content of hormones (Taha, 2017).

Effect of Different Concentration BA, NAA in the Percentage of Callus Induction

The concentration of growth regulators had a significant impact on callus induction frequency of Datura innoxia; the highest significant callus induction frequency 56.75±1.68% was observed on MS media supplemented with 1 mg/l NAA and the lowest was 0.0% with 0 mg/l NAA supplementation (Table 1). There was also significant role of BA on callus induction, supplementation of 1mg/l BA showed maximum callus induction ant that was 56.25±1.89% while the lowest rate was 24.75±1.83% at 0 mg/l of BA supplementation. The effect of the overlap between growth regulators had a significant effect on callus induction, the highest rate 83.0±4.17% at 0.5 mg/l NAA and 1 mg/l BA and the lowest was 27.0±3.14% at 0.5 mg/l NAA and 0 mg/l BA which is very similar result with previous reports (Waoo *et al*., 2013).

Effect of BA and NAA Concentration on Fresh and Dry Weight of Callus

The highest fresh weight for callus was observed 200.61±1.40 mg at 1 mg/l NAA, which differed significantly from other treatments. The concentration of BA had a significant effect on fresh weight of callus, the highest weight 234.54±3.51mg was recorded at concentration 1 mg/l BA, while 1.5 mg/l of BA given a lowest weight 78.41±2.55 mg, which was not different from control. The combinations between growth regulators, highest fresh weight of callus were 395.63±8.58 mg at 0.5 mg/l NAA and 1mg/l BA while fresh weight was significantly reduced to 72.40±2.5 mg at 2 and 1.5 mg/l for both NAA and BA respectively (Table 2). NAA and BA and their combinations had also significant role in dry weight of callus, the highest dry weight of callus was observed 17.73±2.06 mg in 1mg/l NAA and the lowest dry weight reached to 13.18±1.56 mg at 2 mg/l NAA (Table 3).

Table 1: Effect of BA, NAA (mg/l) on callus induction from stem cut explants of *Datura innoxia*

NAA\ BA	0	0.5	1	1.5	Mean
0	0.0±0.0l	0.0± 0.0l	0.0±0.0 l	0.0±0.0l	0.0±0.0 c
0.5	27.0±3.14 k	42.0± 2.53 hi	83.0±4.17 a	55.0±4.32 ef	51.75±0.83 b
1	33.0±4.11 j	59.0± 1.25 de	74.0±3.58b	61.0±5.25 d	56.75±1.68 a
2	39.0±1.32 i	50.0± 1.28 fg	68.0±3.61 c	46.0±3.21 gh	50.75±1.00 b
Mean	24.75±1.83 c	37.75±1.03 b	56.25±1.89 a	40.5±2.94 b	

Notes: Mean values were taken from average of five replications. Mean values followed by the same letters in each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple rang test.

Table 2: Effect of different concentrations of BA and NAA mg/l on the fresh weight (FW) of callus

NAA\ BA	0	0.5	1	1.5	Mean
0	0.0±0.0i	0.0 ±0.0i	0.0±0.0 i	0.0±0.0 i	0.0±0.0 c
0.5	99.54±4.80 g	113.68 ±7.45f	395.63±8.58 a	79.99±3.29 h	172.21±2.41 b
1	108.10 ±4.11fg	244.41 ±4.25C	288.69±4.88 b	161.27±6.25 e	200.61±1.40 a
2	111.78 ±8.47 f	200.03±6.28 d	253.84±4.85 c	72.40±2.5 h	159.51±2.21 b
Mean	79.85±3.47 c	139.53±3.53 b	234.54±3.51 a	78.41±2.55 c	

Notes: Mean values were taken from average of five replications ($n = 5$). Mean values followed by the same letters in each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple rang test.

Table 3: Effect of different concentrations of BA and NAA mg/l on the dry weight (DW) of callus

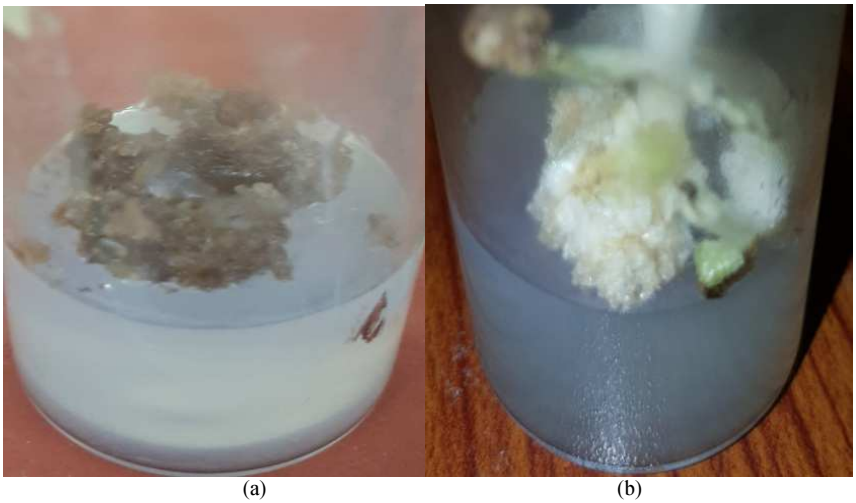
NAA\ BA	0	0.5	1	1.5	Mean
0	0.0±0.0 J	0±0.0 J	0±0.0 J	0±0.0 J	0.0 ±0.0 d
0.5	10.08±4.35 h	10.84±2.53 g	35.64±1.27 a	7.05±3.21 i	15.90±1.29 b
1	10.65±6.25gh	19.51±4.15 d	28.01±4.11 b	12.75±1.23 e	17.73±2.06 a
2	11.90±5.62 f	13.47±2.53 e	20.44±4.28 c	6.93±3.11 i	13.18±1.56 c
Mean	8.15±2.81 c	10.95±1.71 b	21.02±2.12 a	6.68±1.55 d	

Notes: Mean values were taken from average of five replication ($n = 5$). Mean values followed by the same letters in each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple rang test.

Table 4: Effect of different concentrations of BA and NAA (mg/l) on regeneration from stem cut derived callus

NAA\ BA	0	0.5	1	2	Mean
0	0.0±0.0 J	0.0±0.0 J	6.0±1.32i	10.0±1.11hi	4.0±0.70 d
0.5	0.0±0.0 J	13.0±1.11 gh	28.0±3.11 ef	33.0±1.27 de	18.5±1.29 C
1	0.0 ±0.0 J	38.0±1.53 d	78.0±1.55 a	45.0±2.36 c	40.25±0.98 a
1.5	0.0 ±0.0 J	26.0±1.97 F	54.0±5.25 b	17.0±4.12 g	24.25±2.01 b
Mean	0.0 ±0.0 c	19.25±0.84 b	41.5±1.35 a	23.75±1.39 b	

Notes: Mean values were taken from average of five replication ($n = 3$). Mean values followed by the same letters in each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple rang test.



(a) (b)

Fig. 1: *In-vitro* callus induction from different explants of *Datura inoxia* (a) leaf cut explants (b) stem cut explants

Fig. 2: *In-vitro* regeneration of multiple shoots from calli of *Datura inoxia*

The highest dry weight was recorded 21.02±2.12 mg at 1mg/l BA, while the lowest dry weight was 6.68±1.55 mg in 1.5 mg/l from the same regulator. The combination of NAA and BA resulted in a significant increase in dry weight; the highest dry weight was 35.64±1.27 mg at 0.5 and 1 mg/l for both NAA and BA supplementation respectively, the weight reached its lowest and significantly, 10.08±4.35 mg in concentration 0 and 0.5 mg/l for BA and IAA, respectively.

Effect of BA and NAA Concentration on Percentage of Regeneration

BA and NAA also played a significant role on plantlets regeneration. The highest rates of plantlets were observed 40.25±0.98% at 1 mg/l NAA and the lowest 4% at 0 mg/l NAA. 1 mg/l BA showed the highest 41.5±1.35 % plantlets regenerations, while the 0 mg/l of BA gave the lowest 0.0% regeneration (Fig. 2). The combination of NAA and BA had an effect in increasing the rate of plantlet production, the significant highest regeneration found 78% at 1 mg/l when supplementation the media with both BA and NAA, respectively which is similar with previous reports (Jha and Pandey, 2012) (Table 4).

Discussion

This study demonstrated the efficient callus induction and regeneration of medicinally important plant Datura innoxia. The observations showed the maximum callus induction when stem cut explants used and inoculated cultures maintained in dark condition. Treatment with different concentrations and combination of plant regulators showed the different effects. In absence of NAA the cultured media did not induced callus although BA presence in media at different concentrations. The results also showed that the low concentration of NAA was the most effective in the callus induction from explants. This may be due to the fact that high concentration has led to reduced division rates (Taha, 2016), the addition of NAA to the media at highest concentration of the ideal, may adversely affect the function of enzymes responsible for the construction of the cell wall, which affecting mechanical properties and influencing the cell division and formation of callus (Taha, 2017). Addition of both growth regulators to culture media is essential for callus induced, as cytokinein acts with the presence of auxin as a key to initiating cellular division. The response of explants differences due to the ratio of auxin to cytokinein, may be due to differences in the content of these internal parts of the internal hormones, which in turn affects the ideal concentration in the callus induction from auxin or cytokinein or both when added to culture media (Sharma *et al*., 1993; Taha, 2017). The addition of auxin to the MS media higher than the ideal level may adversely affect the work of enzymes responsible for the construction of the cell wall, which affects the mechanical properties of it and influence of cells division and the formation of callus and therefore the weight of fresh and dry weight of callus (Taha, 2017). Increasing the concentration of BA until reached the ideal concentration led to increase the fresh weight of callus, may be due to the fact that cytokine had significant effects in increasing the cells division, especially the meristematic cells and this in turn leads to increase the size of different tissues of plant organs, whether connected to the mother plant separated which cultured in the media (Patel and Rajesh Patel, 2013; Bakar *et al*., 2014; Taha, 2017). Regeneration of plantlets from stem cut explants were done on MS media supplementing the hormones BA and NAA. Different regeneration percentages were observed with different treatment. The reduced percentage of regeneration with increasing the concentration of BA due to an increase in the concentration of BA which may lead to accumulation in the vegetative part as well as its content and therefore its concentration to a level of inhibition, the increased concentration of cytokinins in the culture medium may reduce the number of axiliary branches (Taha, 2017). The reasons of increasing the percentage of regeneration in the combination treatment due to the role played by the balance between the concentration of growth regulators in the renewal of the pattern of tissue differentiation and the formation of organs *in vitro*. Auxin also stimulates genes that cytokinin controls its gene expression and gene expression products play a key role in biological processes such as cell division, chloroplast development and metabolism of nutrients (Taha, 2017).

Conclusion

The present study has shown the successful callus induction from stem cut explants and plantlets regeneration from stem cut derived callus of *Datura innoxia* using different concentrations and combinations of PGRs. The highest rate of callus was observed in MS media when supplemented NAA at 0.5 mg/l and BA 1 mg/l in the dark condition. This combination also gave the highest fresh and dry weight of callus. Highest regeneration of *Datura innoxia* from stem cut derived callus were observed in MS media when supplemented with NAA and BA at 1 mg/l for both.

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Author's Contributions

Baan Munim Twaij: Conceived, designed, performed the experiments and wrote the draft manuscript.

Alaa Jabbar Taha: Data evaluations and validation.

Md. Nazmul Hasan: Analyzed the data and wrote the final manuscript.

Ethics

This article is original and contains unpublished materials. I'm Baan Munim Twaij-the corresponding author confirms that all of the authors have read and approved the manuscript

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