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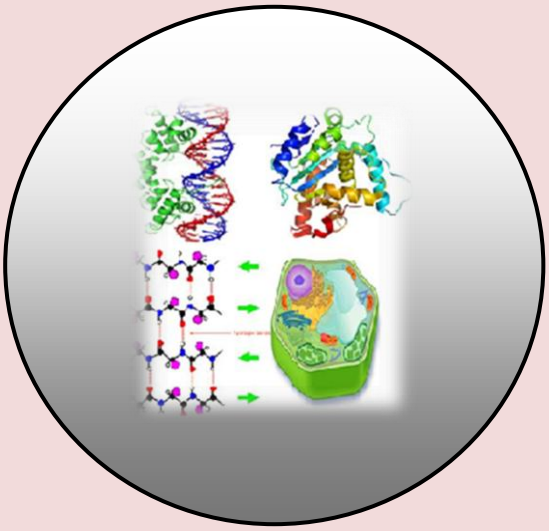

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RESEARCH PAPER

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In-vitro Morphogenesis of *Morus alba* an Important Medicinal Plant Species

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ABSTRACT

Mulberry (Morus alba L.) is an ornamental, medicinal, and fruit plant that belongs to the Moraceae family. One of the most important techniques used in plant biotechnology is tissue culture, which enables mass production of pathogen-free plants. Cotyledon has a high potential for shoot regeneration; however, to the best of our knowledge, there are no reports on plant regeneration from cotyledon explants of mulberry. Therefore, this study aimed to evaluate the potential of seedling-derived cotyledon segments to obtain shoot multiplication of mulberry. Various concentrations (0, 0.25, 0.5, 1, 2, 3, and 4 mg/l) of thidiazuron (TDZ) in combination with indole butyric acid (IBA) were used in a completely randomized design in three replications. The results showed that the highest percentage of regeneration frequency (96.67%) and the maximum number of shoots (4.43) were obtained on Murashige and Skoog (MS) medium supplemented with 0.25 mg/l TDZ and 0.025 mg/l IBA. In the rooting experiment, the maximum rooting percentage (83.33%) and the maximum number of roots per shoot (4.36) were obtained on MS medium containing 2 mg/l IBA. In vitro-raised plantlets were placed in pots and kept in room temperature for 30 days, and the plantlets showed more than 90% survival rate. On the basis of our results, the protocol described in this study has a high potential to be used in the micropropagation of this valuable plant.

Keywords: Mulberry, Cotyledon, Plant growth regulator and Medicinal.

INTRODUCTION

Mulberry (*Morus spp.*) is an important woody feed crop for silkworm in sericulture industry in China, Japan, India and other sericultural important countries. The description of genus *Morus* in the herbarium of Linnaeus (1747) and the work of Draakestein and Van (1678)

highlight the long association of mulberry with Indian sub-continent. Though a large number of species are generally cited in the literature (Koidzumi, 1917; Datta, 2000; Sanchez, 2000), many of the species can be cross fertilized without causing significant seed sterility (Das & Krishnaswami, 1965; Dwivedi *et al.,* 1989).

Morus spp. (Family; Moraceae) commonly known as mulberry plant has several uses for its hardwood (Burkill, 1935), high protein content of the leaf (Pearson & Brown, 1932; Howard, 1948) and also for the presence of specific alkaloids in the leaves, bark and roots (Heilbron & Bunbury, 1953; Datta, 2000). Mulberry foliage as forage, for its high protein content, is also being used traditionally in extensive areas of Africa, Latin America and Asia (Armand & Meuret, 1995; Kitahara, 1999) and renewed emphasis is given internationally on this aspect. The mulberry plant is also known for its importance in tea and coffee plantations as a shade tree and its fruits is also utilized in many places (Facciola, 1990; Reich, 1991) and its importance is obvious as indicated by the web page posted by California Rare Fruit Growers.

Morus alba L. vars. Chinese White, Kokuso-27 and Ichinose are promising genotypes for sericulture industry in temperate regions of the world. In India, these varieties are cultivated for commercial purposes in Jammu and Kashmir, West Bengal, Karnataka and Tamil Nadu (Dandin and Sengupta, 1988; Rajan *et al.,* 1992). Possibility of genetic improvement of Mulberry is very slow and hampered by many factors like it's out breeding tendency, natural intervarietal hybridization and wide range of genetic variation within the species (Gulab Khan Rohela 2020). Advancement in protoplast technology and regeneration procedures has opened new horizons for genetic improvement through protoplast transformation and somatic hybridization (Chatterjee et al., 2004, Yuan et al., 2015).

These techniques may produce the novel genotypes with advance characters like higher suitability to silkworm rearing, better resistance to abiotic factors such as high alkalinity and salinity, water stress, with higher yield (Zhang et al., 2016; Guangqun Ma 2022). This is all depends upon the possibility of development of a suitable method of plant regeneration from protoplast (Thirugnanasambandham et al., 2015).

The silkworm, *Bombyx mori* feed upon the leaves of Mulberry plant. Enzyme urease from host plant is responsible for nitrogen metabolism in the insect body; it incorporated in haemolymph of insect larval stage. For the investigation of absorption of host urease into larva, crude urease from roots and leaves of mulberry was prepared and analyzed electrophoretically. After electrophoresis of urease it was concluded that-

(1) The urease activity appeared in the same migration position in a native gel;

(2) There was no difference in molecular mass of the subunit.

The root urease was orally injected to the fifth instar larvae of the silkworm.

Medicinal Value and Applications of Mulberry

The plant is reported to be used as folk medicines for the management of various diseases like controlling high blood pressure, atherosclerosis, antioxidant, Insulin resistance, Type 3 Diabetes and weight loss etc. (Zhang et al., 2019: Sheng et al., 2018).

During the Last decade the description of genus *Morus* in the herbarium of Linneaus (1747) and the work of Draakestein and Van (1678) highlights the long association of mulberry with Indian sub-continent. Though a large number of species are generally cited in the literature (Koidzumi, 1917; Datta, 2000; Sanchez, 2000), many of the species can be cross fertilized without causing significant seed sterility (Das & Krishnaswami, 1965; Dwivedi *et al.,* 1989).

The first published report on mulberry protoplast fusion and tissue culture was appeared around 45 year earlier (Ohyama and Oko, 1975), however a little studies has been carried out for the regeneration of mulberry plant. The published articles discussed about the challenges of success of this technology (Ohnishi and Kiyama, 1987; Katagiri, 1988; Tohjima *et al.,* 1996) and reported the formation of colonies (Katagiri, 1989; Tewary and Lakshmisita, 1992; Tewary *et al.,* 1995, Rongli Mo et al., 2022), but not succeeded to regenerate plants.
Likewise other medicinal plant, Mulberry is a highly heterozygous plant and shows diverse sex behavior. Mulberry is dioecious so that the breeding work in mulberry is affected heavily.

MATERIAL AND METHODS

Stock Solution Preparation

- **Macro Stock Solution:**

NH_4NO_3	16.5 g
KNO_3	19.0 g
$CaCl_2$	4.4 g
$MgSO_4.7H_2O$	3.7 g
KH_2PO_4	1.7 g

Dissolve in 1000 ml Distilled water

Use 100ml/litre

- **Micro Stock Solution**:

$MnSO_4.4H_2O$	2.23 g
$Zn\ SO_4.7H_2O$	0.86 g
H_3BO_3	0.62 g
KI	0.083 g
$Na_2MoO_4.2H_2O$	0.025 g
$CuSO_4.5H_2O$	0.0025 g
$CoCl_2.6H_2O$	0.0025 g

Dissolve in 100ml of Distilled Water

Use 1 ml/litre

- **Vitamin Stock Solution** :

Thiamine HCl	5 mg
Pyroxide	25 mg
Nicotinic acid	25 mg

Dissolve in 50 ml of Distilled water

Use 1ml/litre

- **Meso-inositol Stock Solution:**

1 g dissolves in 20 ml of Distilled water

Use 2 ml/litre

- **Glycine:**

20 mg dissolve in 10 ml of Distilled water

Use 1 ml/litre

- **Iron stock solution:**

Na_2EDTA 746 mg
$FeSO_4$ 556 mg
Dissolve in 100 ml of Distilled water

Use 5 ml/litre

Step 1: Na_2EDTA dissolve in 40 ml Distilled water
Step 2: $FeSO_4$ dissolve in 40 ml distilled water separately, then mix them in 1 & 2 and finally maintain the volume upto 100 ml.

- **Harmone stock solution:**

Kinetin 5 mg
Dissolve in 10 ml Distilled water
BAP 5mg
Dissolve in 10 ml Distilled water
2, 4 D 5mg
Dissolve in 1N NaOH

Biological Material

Plant Material

The plant material of *Morus alba* (var. Chinese White, Kokuso-27 and Ichinose) collected from 10-year-old trees, maintained in the mulberry Central Sericulture Research and Training Institute. Jammu, India.

Methods

Firstly MS Media is prepared for 1000 ml.
Prepare the stock solution and use according to their composition.
Preparation of MS Media: For 1000 ml MS media the following salt are used as follows:

a) Macro Salt Solution-

Dissolve in 1000 ml Distilled water
Use 100ml/litre.

b) Micro Salt Solution-

Dissolve in 100 ml of D.W
Use 1 ml/litre.

Sterilization

Sterilization of explants, medium and all the glass ware carried out properly. Fully expanded leaves (1.5-2.5 cm), petioles (1 cm) and internodes segments (1 cm) were excised from actively growing branches. The leaves were washed with running tap water and then immersed in 0.5% (w/v) Bavistin solution for half an hour, and then bathed 3-4 times with distilled water.

Explants were then surface sterilized with 70% (v/v) ethanol for 30 sec, followed by 15 min in sodium hypochorite solution, containing 2-3 drops of Tween-80 and were rinsed 4-5 times with sterilized distilled water. The MS basal media (Murashige and Skoog, 1962) was supplemented with various concentration and combinations of growth regulators, with 0.8% (w/v) agar and 3% (w/v) sucrose.

The pH of the media was set to 5.8 and media was autoclaved at 121°C for 15 min at 15 psi. All cultures were incubated at 28±2°C under a 16-h photoperiod with a light intensity of 30 μmol m^2/s^1 provided by cool white fluorescent tubes.

After 8 week of culture, calluses were transferred onto the regeneration media with or without growth regulators. The effect of different concentrations of cytokinns (0.1-5 mg /l BA and kinetin) and auxins (0.1- 2.0 mg/l NAA, IBA and IAA) were tested on shoot morphogenesis. Data were taken as the percentage of calluses forming shoots and the number of shoots formed per callus.

Nodal explants (1-1.5 cm) from *in-vitro* formed shoot were excised with an axillary bud and cultured for rooting. Different auxins (IAA, IBA, and NAA) were used for rooting. The rooted shoots were transferred to the plastic cups filled with a mixture of sand and vermiculite in equal amount (1:1). For high humidity plantlets were covered with polythene bags and placed in shade.

RESULTS AND DISSCUSION

RESULTS

Callus frequency cell division (CF), defined as the dedifferentiated loose cell mass capable of undergoing frequent cell division callus, after culturing of the explant. 10 replicate of each treatment on MS media fortified found to with auxin combination of growth regulator were used and collected from each replica and then % of callus frequency has been calculated.

Table 1. Effect of auxin on callus induction at different concentration cultivated on MS Agar medium.

GR	Concentration (mg/l)					
2 4, D	0.5	1.0	1.5	2.0	2.5	3.0
Callus Frequency (%)	1±2	1.2±1	3±1	3.5±1	3.5±1	3.2±1
NAA	0.5	1.0	1.5	2.0	2.5	3.0
Callus Frequency (%)	1±2	1.2±1	3.0±1	3.0±2	4.1±2	4.3±1

CF = callus frequency

The % callus frequency is maximum in case of 2.0 and 2.5 concentration of 2,4 D mg/l at this concentration the % callus frequency remains constant i.e., 3.5±1 and then when as we increase the concentration there is decrement in the callus frequency. So it shows that callus frequency is max at 2.0 and 2.5 mg/l of 2 4, D. While in case of NAA the best result of callus frequency is obtained at 3.0 mg/l concentration of NAA. As we increase the concentration the callus frequency increases and at 1.5 and 2.0 the callus frequency remains constant and then again increases as we increase the concentration of NAA the percentage callus frequency also increases.

Table 2. Effect of growth regulators on callus induction from mature leaf explant.

Growth Regulators (mg/l)		Shoot induction in Mulberry	
2,4 D + BAP		No. of shoots Length of shoots (cm)	
0	0.5	2	1.5 cm
0.5	1.0	3	2 cm
0.5	1.5	3	2.1 cm
0.5	2.0	4	5 cm
0.5	2.5	2	3.3 cm
IAA + BAP			
0	0.5	1	2.6 cm
0.5	1	3	6.0 cm
0.5	1.5	4	3.9 cm
0.5	2.0	5	7.4 cm
0.5	2.5	2	5.8 cm
NAA + BAP			
0	0.5	2	3.2 cm
0.5	1.0	2	4.8 cm
0.5	1.5	3	3.8 cm
0.5	2.0	4	7.5 cm
0.5	2.5	3	5.5 cm

At constant concentration of growth regulators with combination of BAP in increasing concentration. In case of 2 4, D the best result were obtained at 2 mg/l and the length of shoot was 5 cm. In IAA agar at 2.0 mg/l concentration of BAP the best was obtained i.e. length of shoot was 7.4 cm. Further in case of NAA the best result was at 2.0 mg/l concentration of BAP i.e. the length of shoot was 7.5 cm.

All these data reveals that at constant concentration of growth regulators the 2.0 mg/l is best with combination of BAP gave the good result.

Table 3. Effect of Cytokinin on shoot formation from callus.

Cytokinin	Concentration mg/l	Callus forming shoots	No of shoots per callus
BA	0.0	0.0	0.0
	0.5	21	1.2
	1.0	78	8.2
	1.5	45	3.0
	2.0	56	1.6
Kinetin	0.5	0.0	0.0
	1.0	0.0	0.0
	1.5	12	1.4
	2.0	14	1.0

At 1.0mg/l concentration of BA the callus forming shoots gave good results and at this concentration the number of shoots per callus was 8.2. While in case of kinetin at 1.5 mg/l concentration the no of shoots per callus were obtained was 1.4. So the cytokinin used were gave the good results as we increases the concentration of cytokinin and then no. of shoots per callus decreases as we decrease the concentration of cytokinin.

Table 4. Effect of auxins on root formation under the cultivation of MS Medium with different auxins.

Auxin	Concentration Mg/l	% explant forming roots	Root length (cm)
None	0.0	0	0
IAA	0.5	22	1.5
	1.0	40	1.4
	2.0	58	1.5
IBA	0.5	82	1.4
	1.0	68	1.2
	2.0	70	1.1
NAA	0.5	80	1.2
	1.0	68	1.6
	2.0	70	1.1

Callus initiation occurred from the cut surface of the explant within one week on auxin supplemented media, out of four auxins tested, 2, 4 D was found best auxin for induction of callus. Addition of BA (0.5 mg/l) in the medium containing 2, 4-D enhanced the callus induction response. The maximum explants forming callus on this medium was 100%. While the highest concentration of BA inhibited callus formation from different explants. Variation in callus forming ability of different explants types has been reported in *Morus alba*. Maximum callus initiation is recorded in IAA 0.5 mg/l, and 2, 4 D 0.5 mg/l. Cytokinin type and concentration had a significant effect on shoot differentiation. A was more effective than kinetin for shoot induction. Best shooting response in terms of percent of callus forming shoots 82% and number of shoots formed per callus 8.2 was obtained on the medium supplemented with mg/l BA. High concentration of BA decreased the shoot formation response. Addition of auxins inhibited the morphogenic response and increased callus proliferation. The shoot forming ability of callus culture of *M. alba* has been reported to decrease with high concentration.

a) Callus obtained from MS medium

b) Shoot regeneration from callus

c) Regeneration of shoot bud from callus culture

d) Callus forming shoots

e) Explants forming shoot on MS medium (shoot induction in mulberry)

f) Hardened plant established in soil.

DISSCUSION

Auxins (2, 4 D, IAA and NAA etc.) are routinely employed for the tissue culture studies in mulberry plant. The above results indicated that a precise mechanism and action of auxins are required to induce cell division mulberry plant and is different from other auxins.

Explants were used for the induction of callus, cultivated on MS agar. Medium fortified with auxin. In principle auxins shows tremendous effect on callus induction depends on its concentration and duration of treatment and explants nature. In these studies the same result has been noticed. Similarly for the perforation of callus the same treatment has been repeated during sub culturing process. Combination of auxin and cytokinin showed same better result that has been reported by many scientist earlier the same consequence has been observed in this studied. Auxins are responsible for many activities in plants *viz.* initiate or promote cell division from tissues culture *in-vitro*, apical dominance regulation, control vascular system differentiation, delay senescence, fruit setting and ripening, promote flowering, and stimulate shoot growth and inhibit root growth. Different ratio of auxin to cytokinin is used in morphogenesis. Ratio of auxin to cytokinin is responsible for the shoot or root induction in callus. Higher auxin to cytokinin ratio is root promoting while vice versa is shoot promoting (Lang and Kohlenbach, 1975; Taha et al., 2020). In the same consequences, shoot induction by raising the auxin concentration and its inhibition by the addition of kinetin, has been used (Sastri, 1963). Auxin works as plant growth regulators only in tissue and organ culture in small quantities. Higher concentration of 2, 4 D is inhibitory to plant growth. In present study, plant growth regulators singly (2, 4-D, BAP, Kinetin, NAA and IAA) and in different combinations (2, 4-D + BAP, IAA + BAP, NAA + BAP) were used. The best results were obtained from the combination of NAA + BAP. The possible reason may due to the application of higher concentration of both auxin and cytokinin. Molecular explanation of this phenomenon is enhanced RNA synthesis (Vasseur, 1979). Production of stage specific metabolites may be exploited from *in-vitro* morphogenesis of grown culture of *Morus alba* by pharmaceutical companies. They can produce such metabolites throughout the year from tissue culture grown plant material like medicine for suppression of atherosclerosis.

Rooting

Around 3 cm plantlets were used further rooting purposes from the *in-vitro* grown seedlings by using IBA, NAA and 2, 4 D with semi solid agar medium. For all the cases results were not similar in the case of IBA a vigorous rooting tendency were found to be induced just after 1 week of inoculation. It reflects that the role of IBA is very much transparent in rooting of *Morus alba* (Sarkar et al., 2017). The similar results have been reported for many plant species in the literature such as (Alah *et al.,* 2007). *In-vitro* plant tissue culture can be used for the manufacturing of large-scale propagules for the mass propagation that can help to faster the afforestation programme of the forest department (Sarkar et al., 2022).

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