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Implementation of Tissue Culture Technique for the Production of Triploid Plants of Mulberry (*MORUS. L.*)

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Abstract: ***Mulberry is an important plant in sericulture industry as the foliage constitutes the chief feed for the mulberry silkworm Bombyx mori* (L.) *Various breeding methods which could be profitably adopted for developing superior mulberry varieties. Leaf yield potential and quality of mulberry leaves are greatly influenced by genotypes. The triploid varieties recorded highest nutritive values and produced more leaves than the diploid and tetraploid mulberry plants. Tissue culture technique is suitable for the production of triploid genotypes. Regeneration of plants from endosperm tissue provides an easy and direct approach to triploid production.***

Keywords: Mulberry, Triploid, Tissue culture, Endosperm culture

1. Introduction

Sericulture is one of the important agro based industries, which can cater the needs of agricultural based families of our rural India. Mulberry leaf is soul food for mulberry silkworms the nutritive value of a mulberry leaf has its influence on the growth and development of silkworm *Bombyx mori* (L.) attempts are being made i.e. breeding, hybridization, tissue culture for the production of nutritive mulberry leaf.

Mulberry is an important plant in sericulture industry as the foliage constitutes the chief feed for the Mulberry silkworm *Bombyx mori* is a monophagus insect feeds only on the mulberry leaves. The growth, development, yield, quality and disease resistance in silkworms are mainly depend on nutritive values of the leaf (Ito 1961 a, 1961b, Horie, 1980; Haque et.al, 1990; Krishnaswamy et.al, 1991).

Both natural and in-vivo-induced triploids of mulberry have been reported (Das et al. 1970; Katagiri et al. 1982; Dwivedi et al. 1989) and many of the triploid lines have proved superior to the diploids especially in yield and nutritive qualities of leaves (Seki and Oshikane 1959) and in cold and disease resistance (Hamada 1963). The triploids of many other crops, particularly where the economic product is other than the seed, have proved superior to their diploid or tetraploid counterparts (Bhojwani and Razdan 1996). The conventional method of triploid production, by crossing a diploid with artificially induced tetraploids, is lengthy and tedious. However, regeneration of plants from endosperm tissue provides an easy and direct approach to triploid production (Bhojwani and Razdan1996).

Sugars and Proteins are major elements responsible for the silkworm growth, development and silk production. The silkworm utilizes sugars as source of energy for the synthesis of lipids and amino acids (Horie, 1978, Ito 1960) emphasized that among 20 tested sugars. Sucrose strongly stimulated the feeding behavior followed by Fructose and Raffinose. Nutritional value of proteins is very important as silkworm larvae utilize the leaf nitrogenous matter for their growth and development and synthesis of silk protein (Horie 1978).

Triplod mulberry have been reported (Das et.al 1970: Katagiri et. al 1982: Dwivedi et.al 1989) and many of the triploid lines have proved superior to the diploids, expecially in yield and nutritive qualities of leaves (Seki and Oshikene 1959). The triploids of many other crops particularly where the economic product is other than the seed have proved superior to their diploid and tetraploids. Regeneration of plants from endosperm tissue provides an easy and direct approach to triploid production.

2. Materials and Methods

Plant material: Unmatured fruits [16-17 days after pollination (DAP)] of mulberry plants were collected from the mulberry garden of Sri Krishna Devaraya University (Department of Sericulture) Anantapur. Care was taken when collecting the material and the material which was showing systemic fungal and bacterial infections were discarded, plant material was first subjected to a jet flow of tap water the material then was rinsed in a detergent bath and shaken well for 5 minutes. Again the plant material was washed to remove the detergent. Following this step the plant material was rinsed in 70% ethanol and rinsed in sterilized distilled water for three times until the smell of ethanol was lost. Finally the plant material was surface sterilized with 0.1% mercuric chloride for 8 min. after treating with mercuric chloride the plant material was rinsed thrice in sterile distilled water to remove traces of mercuric chloride. The last two steps were done in a sterile area. And then mulberry fruits were surface dried by placing them in sterile Petri dishes lined with blotting paper, and the seed was dissected with the aid of a binocular microscope. The fleshy perianth was removed and the seed coat opened with the help of a pair of needles. The excised endosperm, with or without the embryo, was cultured on Murashige and Skoog (1962) medium containing 3% sucrose and gelled with 0.8% agar.

	Stock	
	Mg/l(40X)	Mg/l
A. Micro nutrient stock		
$Mn.SO_4,4H_2O$	892	22.3
$Zn\ SO_4\ 7H_2O$	344	8.6
$CuSO_4\ 5\ H_2O$	1	0.025
H3BO	248	6.2
KI	33.2	0.83
$Na_2MoO_42H_2O$	10	0.25
$CCl_2\ H_2O$	1	0.025
B. Iron Stock	Mg/l(40X)	Mg/l
Na2EDTA	1494	37.35
$FeSO_4,7H_2O$	1114	37.35
C. Vitamin stock	Mg/l(40X)	Mg/l
Nicotinic acid	40	1.0
Pyridoxine H Cl	40	1.0
Thiamine H Cl	40	1.0
Glycine	16.0	4.0
D. Macro nutrients		
NH_4NO_3		825
KNO_3		950
$CaCl_2$		220
KH_2PO_4		85
$Mg\ SO_4$		185
Inositol		100
Sucrose		20
Agar (0.9/w/v)		20,000

Micro nutrients: Concentrated stock solutions of micro nutrients listed in table were prepared. Iron stock was prepared separately to avoid problem with iron solubility and it was prepared in a chelated from as the sodium salt of Ferric ethylene diamine tetractate.
Vitamins: Thiamine H Cl, Nicotinic acid, Pyridoxine, H Cl were added in media.

Amino acids: Glycine was added to M.S.medium activated charcoal 0.1 activated charcoal was supplemented to the nutrient media as it adsorbs secondary products secreted by cultured tissues.

Carbon source: 20,000 mg/l sucrose was added for M.S. media

Water: Sterilized double distilled water was employed in all tissue culture media, including water used during the culture procedure.

Medium Matrix: 0.9/l Difco bacto agar was used for gelling medium.

3. Preparation of Stock Solutions

Micro nutrient stock: Approximately 50 ml of double distilled water was taken in a 100 ml beaker. Salts were weighted according to the first column of the table-I weighted salts were dissolved separately, solutions was transferred to the 100 ml of volumetric flask and made up to the mark. This micro nutrients stock was stored under refrigeration.

Iron stock: To 50 ml of double distilled water weighed Na2 EDTA was added and boiled to dissolve, weighted FeSO4 7H2O was added to the boiling solutions. After 5 minutes the solution was transferred to the volumetric flask of 100 ml capacity. Double distilled water was added to make the solution to final volume. Iron stock was stored at room temperature.

Vitamin stock: Vitamins are weighed according to the column 1 of table 1 and dissolved in 50 ml of double distilled water. This vitamin mixture was transferred to the 100 ml volumetric flask and double distilled water was added to the final volume. Vitamin stock was stored under refrigeration.

2, 4-D stock: 10 mg of 2, 4-D was dissolved by adding 2, 3 drops of ethanol, few ml of double distilled water was added and the transferred to volumetric flask. This was made up to 100 ml by adding double distilled water.

Kinetin stock: 10 mg kinetin was dissolved in few drops of 1N HCl, about 10 ml of double distilled water was added and transferred to the volumetric flask kinetin was made up to the final by adding double distilled water.

IAA stock: Indole auxins can be dissolved in 1N NaOH IAA was dissolved in few drops of 1N Na OH and this was transferred to a volumetric flask of 100 ml after adding 10 ml of distilled **water:** Double distilled water was added in, order to make up the solution to the final volumes.

NAA stock: NAA can also be dissolved in Na OH. The same procedure given to the IAA stock was followed to prepare NAA stock.

The hormone stock solutions were stored in refrigerator. All the stock solution was labeled including the concentration and date of preparation. All the stock solutions were used within 30 days and discarded after 30 days.

Preparation of M.S. medium: To 400 ml of double distilled water, weighed macro nutrients were added separately and dissolved. From each of the previously prepared stock solutions (Micro nutrient Iron and Vitamins) 2.5 ml was added 0.5 ml of 2, 4-D stock solution was added 20,000 mg sucrose was added. Again 400 ml distilled water was added, Ph of the medium was adjusted to 5,6 by delivering drop lets of 1N Na OH or 1N H Cl with separate pipettes 0.1% activated charcoal and 0.8% agar was added. After adjusting the PH the medium was made up to liter by adding double distilled water.

Medium was boiled to dissolve Agar. After the complete dissolution of Agar 15 ml of medium was dispensed into culture vials culture vials were tightly closed with Aluminum foils. The media was sterilized in an autoclave for 15 mints at 151 lb/in2 at the end of sterilization period the pressure was permitted to return slowly to zero. After removing the culture vials from the autoclave they were placed in a slant potion on a sterilized bench and these were left for a day. As the medium comes to room temperature agar gets solidified forming a slant.

Aseptic techniques: The nutrient media would support a luxuriant growth of many micro organisms, like bacteria and

fungi. To avoid catastrophe due to micro organisms rigorous asepsis and cleanliness was adopted during the entire process. The invasion of micro organism may be at 3 stages.

They may be present right from the beginning in the media or they may enter while preparing or dispensing the media. The entry of such micro organisms in to culture foils. Micro organisms already existing in the media and enter during dispensing were destroyed by autoclaving at 120^{O} C (15 lb/in^2) for 15 min. Incubation: The room for incubating cultures was maintained at a controlled temperature cultures were maintained at 26 ± 2^{o} C under white fluorescent light with 16/8 hr. photo period.

Sub culture: Callus induced on the medium was allowed to grow initially for 20 days in order to establish and maintain the callus. The callus was transferred on to a freshly prepared medium containing same composition containing 1 mg/l Kn+1 mg/l IAA plantlets were well developed these plantlets were transferred to tooting medium liquid M.S. medium fortified with 2 mg/l IAA was used as rooting medium. The shoots regenerated from endosperm callus were multiplied on MS medium supplemented with BAP or Kn. For rooting, the individual shoots, measuring about 4 cm in height and bearing three to four leaves, were isolated and cultured on M.S. medium

Establishment of plants in soil: In-vitro grown plants were thoroughly washed in tap water. Old leaves were pinched off. Transplanting was done into small pots with autoclaved sand and saw dust. Humidity was maintained around plants. Plants were irrigated 2-3 times during the first 6 days with half strength M.S. inorganic salts. After 10 days plants were acclimatized for natural conditions.

4. Results

The endosperm callus was sub cultured 60 days after culture initiation on the original medium as well as several other auxin treatments, most of the media supported callus proliferation, but based on the number cultures showing sustained proliferation and the degree of callusing, MS with 2, 4-D was the best.

To induced regeneration, the endosperm callus from MS with 2,4-D was sub-cultured on MS medium supplemented with BAP or TDZ alone, or in combination with NAA. There was no differentiation on the basal medium but all cytokinin treatments induced shoot but differentiation, the highest number of culture showed shoot but differentiation on MS medium containing BAP and NAA, followed by the medium containing TDZ. However, in terms of the number of shoots per culture, the latter treatment was the best.

5. Discussion

Endosperm is a unique tissue in its origin, ploidly level and nature of growth. It is mostly formed by the fusion product of three haploid nuclei, one from the male gametophyte and two from the female gametophyte and is, therefore, triploid. Regeneration of plants from endosperm tissue provides an easy and direct approach to triploid production. Chaluvachari and Bongale (1994) also recorded leaf quality variation between the diploid and triploid varieties and also reported that the triploid varieties recorded distinctly highest values of leaf protein, sugars and mineral contents associated with superior rearing performances and bioassay moulting test parameters.

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