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In-vitro Response of M5 and Mysore Local (*Morus spp*) with Different Concentrations of 2,4-D

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Abstract: ***In-vitro techniques such as tissue and organ culture offer the plant breeder new openings in the clonel propagation, medium composition is very important in tissue/organ culture. No single medium can be suggested for all types of plants and organs, so that the details of culture medium need to be worked out for each plant material separately. In the present study nodal explants of M5 and Mysore local (Morus spp.) were used for in-vitro callus initiation ability and evaluating best suitable media for M5 and Mysore local. Increase or decrease in the explants size caused changes in callus formation so explants about 5 mm in size were used for inoculation. The most variable factors in the medium are growth regulators, which may be adjusted by using different concentrations of each. The combination giving best results may be selected. Different concentrations of 2,4-D at 0.5, 1.0, 1.5, 2.0 mg/l were present in the medium***.

Keywords: Tissue culture, Mulberry, Plant hormones, 2,4-D, *In-vitro*

1. Introduction

The beginning of plant tissue culture was made as early as 1898, when a German botanist, G. Haberlandt successfully cultured fully differentiated individual plant cells, isolated from different tissues in several plant species. For about thirty five years (i.e. up to 1934), little further progress in cell culture research was made although culture of embryos, roots and other tissue was achieved in this period. During 1934-1939, due to discovery of the importance of auxins and B-vitamins, the foundation of plant tissue culture was laid down by three scientists (Gautheret, White and Nobecourt), even though only small pieces of tissue and not individual differentiated cells could be grown in cultures.

No single medium can be suggested for all types of plants and organs, so that the details of culture medium need to be worked out for each plant material separately. Several recipes have been developed during the last five decades; the major constituents of a culture medium include Inorganic nutrients, organic nutrients, growth hormones and agar. The growth hormones included culture media involve auxins, cytokinins and gibberellins. The auxins are mainly used to facilitate cell division and root differentiation. In order to find a suitable culture medium for a plant material, one should start with a well known medium, and by a series of experiments, a new medium may be evolved. The most variable factors in the medium are growth regulators, which may be adjusted by using different concentration of each. The combination giving best results may be selected.

pH determines many important aspects of the structure and activity of biological macromolecules. Nutrient medium pH ranges from 5.0 to 6.0 for suitable in vitro growth of explants. pH higher than 7.0 and lower than 4.5 generally stops growth and development.

2. Material and Methods

Plant Material

Two varieties of Mulberry M5 and Mysore local were selected for this investigation. Actively growing shoots from 60 days old (days after pruning) plants were selected. Top 15-20cm long shoots were chosen and 3-4 nodes from top to down were rejected. The selected shoots should be free from any visible signs of diseases and pests. The shoots were cut with a scissor and kept in a conical flask containing water and brought to the laboratory.

The explants were washed in running tap water to remove soil and dust for 30 minutes, then leaves were removed, after removing the leaves, the stem was washed with sterile double distilled water for 2 to 3 times, then ex-plant material immersed in 70% ethanol for 60 sec. later it was sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for 30 seconds and washed with double distilled water to remove the traces of chemicals. Sterilized plant material was transferred on to sterilized Petri plate containing filter paper. With the help of forceps and scalpel the explants were dissected and damaged ends were remover blotted on a sterile filter paper. Then explants were inoculated on to culture medium vertically to maintain polarity the mouth of culture tubes were covered with sterilized aluminum foil. Culture tubes were labeled giving the details of the experiment, name of the explants, date of inoculation etc. nodel explants of M5 and Mysore local were used for in-vitro studies. Their callus initiation ability and evaluating best suitable media for M5 and Mysore local (*Morus spp*). Increase or decrease in the explants ex-plants size caused decrease in callus formation so explants about 5 mm in size were used for inoculation. Different concentrations of 2,4-D at 0.5, 1.0, 1.5, 2.0 mg/l were in the medium.

Composition of media (mg/l)

Ingredient	M.S. Medium*	B5 Medium**
NH_4NO_3	1650	--
KNO_3	1900	2500
$Ca(NO_3)_2.4H_2O$	--	--
$CaCl_2.2H_2O$	440	150
$MgSO_4.7H_2O$	370	250
KH_2PO_4	170	--
$Na_2SO_4.10H_2O$	--	--
$(NH_4)2SO_4$	--	134
$NaH_2PO_4 H_2O$	--	150
KCl	--	--
KI	0.83	0.75
H_3BO_3	6.2	3.0
$MnSO_4.4 H_2O$	22.3	--
$MnSO_4 H_2O$	--	10.0
$ZnSO_4.7 H_2O$	8.6	2.0
$Na2MoO_4.2 H_2O$	0.25	0.25
$CuSO_4.5 H_2O$	0.025	0.025
$CoCl_2.6 H_2O$	0.025	0.025
Na_2EDTA	37.3	37.3
$FeSO_4.7 H_2O$	27.8	27.8
Sucrose	30,000	20,000
Inositol	100	100
Nicotinic acid	0.5	1.0
Pyridoxine-HCl	0.5	1.0
Thiamine-HCl	0.1	10.0
Glycine	2.0	--
Kinetin	1.0-2.0	0.1
Indoleacetic acid	0.3-3.0	--

*Murashige & Skoog's (1962)
** Gomborg, Miller and Ojima (1968)

Preparation of media

The formulation of MS and B5 media were represented in table, different concentrations of 2,4-D, IAA, NAA and Kn were used to study the callus initiating abilities and regenerating abilities of nodal explants of M5 and Mysore local. These growth regulators were dissolved in suitable solvent before preparing stock solution.

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

NAA stock:
NAA can also be dissolved in 1N NaOH and this was transferred to a volumetric flask of 100 ml after adding 10 ml of double distilled water and make up the solution to the final volume.

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

Preparation of media:

To 400 ml of double distilled water weighed nutrients (as per table) were added separately and dissolved. From each of the previously prepared stock solutions they mixed as per above mention combination and 0.5 ml of 2,4-D stock solution was added sucrose was also added as per combination above mentioned. The solution was stirred until the sucrose was completely dissolved. Again 400 ml double distilled water was added. pH of the medium was adjusted to 5.6 by delivering droplets of 1N NaOH or 1N HCl which separate pipettes. 0.1% activated charcoal and 0.8% agar was added. After adjusting the pH, the medium was made up to 1 liter by adding double distilled water.

In the same manner the media with different concentration of 2,4-D such as 1mg/l, 1.5mg/l and 2mg/l were prepared.

Sterilization techniques:
The first condition for the success of a culture is asepsis. A few simple precautions to avoid contamination will save valuable time in not repeating experiments. The nutrient media used in tissue culture were sterilized by autoclaving. In present investigation culture media in glass containers sealed with aluminum foil were autoclaved at 15 psi and 121^{O}C for 15 minutes. Glass culture vials were mostly sterilized along with medium whereas the glassware used presterilized nutrient medium preparation was sterilized by dry heating in hot air oven at 160-180 ^{O}C for 3 hrs.

For aseptic manipulation, the instruments such as forceps, scalpels and scissors were sterilized by dipping in ethanol, followed by flaming and cooling, which is referred as flame sterilization.

3. Result

The present study clearly demonstrated the differential behavior of M5 and Mysore local varieties of mulberry. B5 medium supplemented with 2 mg/l was the relatively best suitable medium for callus initiation in Mysore local. Time taken for callus ignition was 6days and 60% of the explants showed callusing on B5 medium supplemented with 2mg/l 2,4-D, whereas on MS medium with same supplement the explants shoed delayed initiation 11 days with less percent frequency 38%. When the variety M5 was inoculated on to the same B5 medium fortified with 2mg/l 2,4-D it showed callus ignition after 11 days in 33% of the explants. When the B5 medium was replaced by MS medium supplemented with 2 mg/l 2,4-D M5 respond well. Higher concentration of 2,4-D induced higher percent frequency of callus but lower concentrations reduced the percent frequency significantly. Time taken for callus induction in different concentrations of 2,4-D was recorded.

Once the shoots were well developed these were transferred to liquid medium for regeneration of roots. Filter paper bridges were used to support the shoots. Different hormones such IAA, 2,4-D and NAA were used. During the first sub-culture only half the number of total shoots showed the

initiation of roots, but during subsequent sub-culturing 100% initiation of roots was noticed. Continuous callus cultures were maintained for about 8 months. Plantlet formation via callus cultures could not be achieved in the present investigation with the use of Kinetin. Plantlets were obtained only from axillary bud cultures. In all the treatments, control cultures (without any hormone) were used and they did not show any response. The ability of the shoots to root was very high very high in liquid medium when compared to the solid medium.

Effect of different concentration of 2,4-D in both media (MS medium and B5 medium)
Mysore local

S. No	Concentration of 2,4-D (mg/l)	MS medium		B5 medium	
		Time taken for callus initiation (Days)	% Frequency of callus initiation	Time taken for callus initiation (Days)	% Frequency of callus initiation
1	0.5	14	12	13	24
2	1.0	13	17	11	36
3	1.5	12	35	09	57
4	2.0	10	43	06	62

M5 verity

S. No	Concentration of 2,4-D (mg/l)	MS medium		B5 medium	
		Time taken for callus initiation (Days)	% Frequency of callus initiation	Time taken for callus initiation (Days)	% Frequency of callus initiation
1	0.5	13	20	17	10
2	1.0	13	31	14	14
3	1.5	10	50	12	29
4	2.0	06	100	11	33

4. Discussion

Oka and Ohyama (1976)[8] reported that the addition of 2,4-D remarkably enhanced the callus formation at the lower part of the explants, but the shoots developed rarely. The effect of different concentrations of 2,4-D (0.5, 1.0, 1.5, 2.0 mg/l) on sprouting, rooting and callus proliferation was studied in the present investigation, high concentration of 2,4-D (2.0 mg/l) showed remarkable suppression of both sprouting and rooting. Gamborg et al. (1976)[4] stated that 2,4-D is a powerful suppressant of organogenesis and it would not be used in experiments involving root and shoot initiation. Though this herbicide is suppressing organogenesis at a concentration of 2mg/l, however, there was 100% sprouting in M5 and 50% in Mysore local at a low concentration (0.5mg/l) of 2,4-D. Rooting from the cut ends can also be seen in both the varieties at this concentration. As the concentration of 2,4-D increased from 0.5to1.5mg/l the percent of sprouting and rooting decreased.

Selection of explants, age of the plant and type of hormones play a key role in in-vitro propagation trees. Methods of plant propagation by in-vitro methods include shoot culture with proliferation of axillary or adventitious shoots and callus culture with regeneration of shoots or embroyoids (Hussey, 1978)[5]. Callus was yellow and smooth in Mysore local, nodular and yellow in M5. During subsequent cultures callus ceased to grow and appeared brown the colour due to accumulation of phenolic compounds. This was reported by Tewary et al. (1989)[11] in mulberry and also in other angiosperic taxa by Shah and Mehta (1976)[9] and Singh et al.,(1982)[10]. By using 0.1% activated charcoal this was prevented in the present investigation.

5. Conclusion

From the present study it can be concluded that the nutritional requirements for getting callus culture and micro propagation can be further exploited for the applied studies and commercial propagation of the two verities undertaken.

6. Future Scope

Nodel explants of M5 and Mysore local were used for in-vitro studies. Their callus initiation ability and evaluating best suitable media for M5 and Mysore local (*Morus spp*). nutritional requirements for getting callus culture and micro propagation can be further exploited for the applied studies and commercial propagation of the two verities undertaken.

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