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Tissue culture studies on embryo axis of *Jatropha curcas* L.

Vetrivel Vadivel and Suresh kumar Jebamalai
PG and Research Department of Botany, Government Arts College
(Affiliated by Thiruvalluvar University, Vellore),
Tiruvannamalai, Tamil nadu, India.

Abstracts

Tissue culture experiments on precursors of this herb will definitely improve to attain a greater improved and increased productivity. Hence, the present study has been undertaken the objectives were selection of explant, induction of callus, root and shoot and hardening of seedlings. The selected medicinal plant was *Jatropha curcas* L. Different concentration and combinations of growth regulators such as IAA, NAA, 2,4-D and BAP were used. It was observed that embryo axis explants showed better growth response like enlargement and initiation of callus. Therefore, only embryo axis explants of *J. curcas* L. were used throughout the present study. Embryo axis exhibited higher growth in the media supplemented with 5mg/l of 2,4-D and NAA; 2 mg/l of IAA and 1mg/l of BAP.

Key words: *Jatropha curcas*, growth regulators, media, proliferation.

INTRODUCTION

The production of alternative fuels can have widespread effects. For example, the production of corn-based ethanol has created an increased demand for the feed stock, causing rising prices in almost everything made from corn. However, in a competitive free market, an increased supply of ethanol reduces the demand for conventional fuels, and thus lowers fuel prices. The ethanol industry enables agricultural surpluses to be used to mitigate fuel shortages[1]. "Biodiesel" is standardized as methyl ester and other diesels of biological origin are not included[2]. Divakara *et al.* (2010)[3] have reported that as a whole it has multiple uses. Hence, there is a worldwide interest in cultivation and improvement of *Jatropha* for biodiesel production. Weak root system is one of the major difficulties in the successful establishment of micropropagated plantlets in field conditions[4].

All parts of *Jatropha* plant have been used in traditional medicine and for veterinary purposes for a long time[5,6]. Some compounds (Curcacycline A) with antitumor activities were reportedly found in this plant[7]. Substances such as phorbol esters, which are toxic to animals and humans, have been isolated and their molluscicidal, insecticidal and fungicidal properties have been demonstrated in lab-scale experiments and field trials[8,9].

The seed oil can be applied to treat eczema and skin diseases and to soothe rheumatic pain[10]. Duke (1988)[11] reported that oil has a strong purgative action and is widely used for skin diseases and to soothe pain such as that caused by rheumatism. The latex itself has been found to be a strong inhibitor to watermelon mosaic virus[12]. The leaves and latex, are used in healing of wounds, refractory ulcers, and septic gums and as a styptic in cuts and bruises. A proteolytic enzyme (curcain) has been reported to have wound healing activity in mice[13,14]. Investigation of the coagulant activity of the latex of Jatropha showed that whole latex significantly reduced the clotting time of human blood. Diluted latex is known to prolong the clotting time and at high dilutions the blood does not clot at all[15].

Mujumdar and Misar (2004)[16] reported that the Bhil tribes from Rajasthan area in India uses *Jatropha* root powder in paste form for the treatment of inflammation. The methanol extract of roots when applied to acute paw edema in mice, exhibited systemic and significant anti-inflammatory activity.

Biodiesel

The seed oil of Jatropha was used as a diesel fuel substitute during the World War II[17]. Engine tests with Jatropha oil were done in Thailand, showing satisfactory engine performance[18]. For African countries, the feasibility of the production of fatty acid ethyl esters from Jatropha oil was studied[19]. The economic evaluation has shown that the biodiesel production from Jatropha is profitable provided the byproducts of the biodiesel production can be sold as valuable products[20].

Jatropha curcas oil has desirable physico-chemical and performance characteristics as diesel[21, 22,23,24]. Additional advantage is that the diesel engine vehicles do not need any modifications for its use. Further, the use of Jatropha biodiesel is eco-friendly without the emission of sulphur and less carbon emission.

Uses in traditional veterinary practices and medicine

Most parts of *J. curcas* have been widely used for veterinary purposes. The seeds are highly effective against *Strongyloids papillosus* infection in goats[25].

Phytochemicals analysis

Jatropha species are rich sources of phytochemicals such as terpenes, cyclic peptides alkaloids and lignans[26]. Numerous papers have reported the presence of secondary metabolites in different parts of *J. curcas*[27,28,29,30,31,32].

Tissue culture

The use of *Jatropha curcas* L. as a source for biodiesel has generated substantial interest in this species[33,34,35]. Owing to its potential, considerable research has been carried out on its phylogeny and physical/genetic characterization which is very essential to domesticate and improve the crop. The phenotypic diversity studies help in classification and identification of accessions to be used for meeting specific breeding objectives.

MATERIALS AND METHODS

Plant materials

Jatropha curcas L.

The major plant source for this study was *Jatropha curcas* L. (Euphorbiaceae), commonly known as biodiesel plant. The immature seeds were collected from the field and then embryo axis excised for further studies.

Methods

Tissue culture studies

Selection of explant

Embryo explants were used for the present study. The first fully matured seeds were selected for this study. The explants were excised with the help of sterile forceps and blade.

Surface sterilization of the explant

Surface sterilization was done by using mercuric chloride and alcohol. The explants were treated with 0.1 per cent mercuric chloride for 1 minute and washed twice with sterile distilled water. Then the materials were rinsed in 50 per cent alcohol for 2 to 3 minutes. Then the explants were thoroughly washed twice with sterile distilled water.

Preparation of medium

Murashige and Skoog's (1962)[36] (MS) medium was used throughout the study (Table-1 & 2).

Table-1: COMPOSITION OF MS BASAL MEDIUM

COMPONENTS	CONCENTRACTION (mg/l)
Macro elements:	
NH_4No_3	1650
KNo_3	1900
$CaCl_2$. $2H_2O$	440
Mg So_4 $7H_2O$	370
KH_2 PO_4	170
Na_2 EDTA	37
$FeSo_4$ 7 H_2O	27
Micro elements:	
H_3 Bo_3	6.2
$MnSo_4$ $4H_2O$	22.3
$ZnSo_4$. $4H_2O$	8.6
KI	0.83
Na_2 Mo O4 $2H_2O$	0.25
$CuSo_4$ $5H_2O$	0.25
$CoCl_2$ $6H_2O$	0.025
Organic Vitamins:	
Glycine	2
Myo – inosital	100
Nicotinic acid	0.5
Pyridoxine HCl	0 .5
Thiamine HCl	0.1
Sucrose	30000
Agar agar	10000

Growth Regulators:

IAA	-	0.5 - 5mg/l
BAP	-	0.5 – 5mg/l
2,4-D	-	0.5 – 5mg/l

NAA - 0.5 – 5 mg/l
pH - 5.5

Stock solutions of major and minor elements including vitamins were prepared by dissolving adequate quantities of each element as follows:

Table-2 Stock solutions

Solution	Solution	Constituents in a stock solution gm / 100ml	Concentration Volume final medium (ml / l)
A	$NH_4 No_3$	8.25	20
B	KNo_3	9.5	20
C	$H_3 Bo_3$	0.124	05
	$KH_2 Po_4$	3.4	
	KI	0.0166	
	$NaMoO_4.2H_2O$	0.005	
	$CoCl_2 6H_2O$	0.0005	
D	$CaCl_2 2H_2O$	8.8	05
E	$MgSo_4. 7H_2O$	7.4	05
	$MnSo_4 4H_2O$	0.448	
	$ZnSo_4 5H_2O$	0.172	
	$CuSo_4 7H_2O$	0.0005	
F	Na_2 EDTA	0.745	05
	$FeSo_4 7H_2O$	0.557	
G	Thiamine Hcl	0.01	01
	Nicotinic acid	0.05	
	Pyridoxine Hcl	0.05	
	Glycine	0.2	

Na_2 EDTA was heated and mixed under constant string with the $FeSo_4 7H_2O$ Solution. Heating and string resulted in a more stable Fe EDTA complex. All the stock solution were stored in glass bottles under refrigeration. The bottle which contained the stock solution was shaken gently prior to use. If any of the solution showed a suspension or precipitate they were immediately discarded. Details about the concentration and date of preparation of all the stock solutions were labeled. The stock solutions were used only for a period of four weeks. Appropriate quantities of the various stock solutions, sucrose, myo-inositol and growth regulators were added. The final volume of the medium was made-up to large volume using double distilled water.

After mixing thoroughly, the pH of the medium was adjusted to 6.0 using 0.1N NaOH or 0.1N HCl. Then appropriate quantity of agar was added. The medium was heated until the agar was dissolved; then poured into the culture tubes (15ml of the medium in 25 X 150mm culture tube) and the tubes were tightly plugged with absorbent cotton. After that the plugged culture tubes were sterilized in a pressure cooker at 121^{o}C for 20 minutes and cooled at room temperature.

Inoculation

Before starting inoculation, culture tubes containing media, instruments like sprit lamp, sterilized forceps, scissors, petridishes and sterilized distilled water were transferred to UV chamber and they were exposed to UV light, for 30 minutes. After that the surface sterilized explants were inoculated. The embryo axis explants were implanted on the medium with posterior end in contact with nutrient medium.

Culture room

The culture room was maintained at a temperature of $25^0 \pm 2^0$C. The cultures were kept under the light intensity of 2,000 Lux at the level of culture tubes, using white fluorescent lamps. Photo-period of 12 hours per day was maintained. The relative humidity of the room was maintained at 70 per cent.

Sub culture

Calli and shoots were taken from the previously grown from the tissue cultured plants and sub cultured every 5-6 weeks. The tubes containing culture materials were externally sterilized with 50 per cent alcohol. The materials were transferred to fresh medium with the help of sterile forceps in the inoculation chamber. After subculture they were transferred to the culture room.

Growth measurement

Fresh and dry weights of the explants were measured before inoculation. Fresh and dry weights of the calli were measured after 5 weeks of culture. Regular observation at an interval of two days was made for the formation of callus, change of colour and initiation of the root or shoot.

RESULTS

Different explants of *Jatropha curca*s L. were cultured on different concentration of hormones (IAA, NAA, 2,4-D and BAP). It was observed that embryo axis explants showed growth response like enlargement and initiation of callus. Therefore, only embryo axis explants of *J. curcas* L. were used throughout the present study.

4.1 Determination of optimal concentration of Hormones

Different concentrations (0.5, 1, 2 and 5 mg/l) of IAA were used, the maximum growth in callus of fresh and dry weight was observed at 5mg/l and minimum growth was observed at 0.5mg/l. The growth responses of IAA were parallel to the concentration (Table-3).

Different concentrations of 2,4-D (0.5,1, 2 and 5mg/l) were used, 5 mg/l was found to be enormously promoting growth from the embryo axis explants. The other concentration of this hormone also induced maximum callus (Table-3).

Different concentrations (0.5, 1, 2 and 5 mg/l) of NAA were used, the maximum growth in callus of fresh and dry weight was observed at 0.5mg/l and minimum growth was observed at 5mg/l. Hormone concentrations were equal to the growth responses which lower concentration induced maximum amount of callus but higher concentrations induced minimum amount of callus (Table-3).

Table-3

Effect of hormones on the embryo axis explants of *Jatropha curcas* L.

S. NO	Morphogenetic Growth Responses						
	IAA	BAP	NAA	2,4-D	Callus	Shoot	Root
1.	0.5	-	-	-	+	+++	-
2.	1.0	-	-	-	+	++	+
3.	2.0	-	-	-	+	+	+
4.	5.0	-	-	-	+	-	-
5.	-	0.5	-	-	+	+	-
6.	-	1.0	-	-	++	+	-
7.	-	2.0	-	-	+	+	-

8.	-	5.0	-	-	+	+	-
9.	-	-	0.5.	-	-	+	+
10.	-	-	1.0	-	-	-	++
11.	-	-	2.0	-	-	+	+++
12.	-	-	5.0	-	-	+	+
13.	-	-	-	0.5	+	+	+
14.	-	-	-	1.0	++	+	+
15.	-	-	-	2.0	+++	+	+
16.	-	-	-	5.0	+	-	-

- - Not response ; + - 20% ; ++ - 40%
+++ - 60% ; ++++ - 80%

Among the four concentrations of BAP (0.5, 1, 2 and 5mg/l) were used, 0.5mg/l was proved most effective than other concentrations. Generally this hormone has very good performance in this study (Table-3).

Callus induction:

All the concentrations of IAA induced callus from the explants but its callulogenetic capacity was very low. Light green coloured callus initiated from the explant after one week, among these all BAP concentrations BAP 1mg/l gave the maximum amount of callus. There was no callus responses from the NAA supplemented media. In 2,4-D treated media induced light brown colour and friable calli from the embryo explants. Calli were initiated from the first week of the inoculation, which were fully formed from the fourth week onwards. In the fourth week, callus was friable and light brown on 2mg/l 2,4-D. The calli were changed into brown colour in the sixth week on the media containing 2mg/l (Table-3).

Shoot induction

IAA 0.5 and 1mg/l respectively containing media developed callus with shoot from the node explant.

Root induction:

1 and 2mg/l of NAA induced callus with roots from the node explants. Generally inoculated node produced callus first and then roots from the developed callus (Table-3).

Effect of factorial combination of IAA and BAP

Factorial combination of different concentration and combination of IAA and BAP was tried to elicit morphogenetic potential of node explants. Friable nature, generally white colour but rarely light brown colour callus was observed on the all combinations of IAA and BAP. 0.5mg/l of IAA and 1mg/l of BAP combinations gave the high callulogenetic responses, which was 1: 2. 0.5mg/l and 1mg/l of IAA with all concentrations of BAP (0.5, 1, 2, 5mg/l) showed only callus (Fig.1).

The maximum amount of callus was originated from the explants on the media supplemented with the IAA 1 + BAP 2mg/l which was wheat coloured callus (Fig.4;Table-4). Shoot induction from the node explants of this plant was observed on the combinations of IAA 1mg/l with 2mg/l BAP and IAA 2mg/l with 5mg/l BAP (Figs.5 & 6). The shoot grew only 1cm but has not elongated (Table-4).

Root induction with callus was originated from media containing IAA amount was higher than BAP. IAA 2 + BAP 0.5 and IAA 5 + BAP 0.5mg/l (Figs.2 & 3; Table-4).

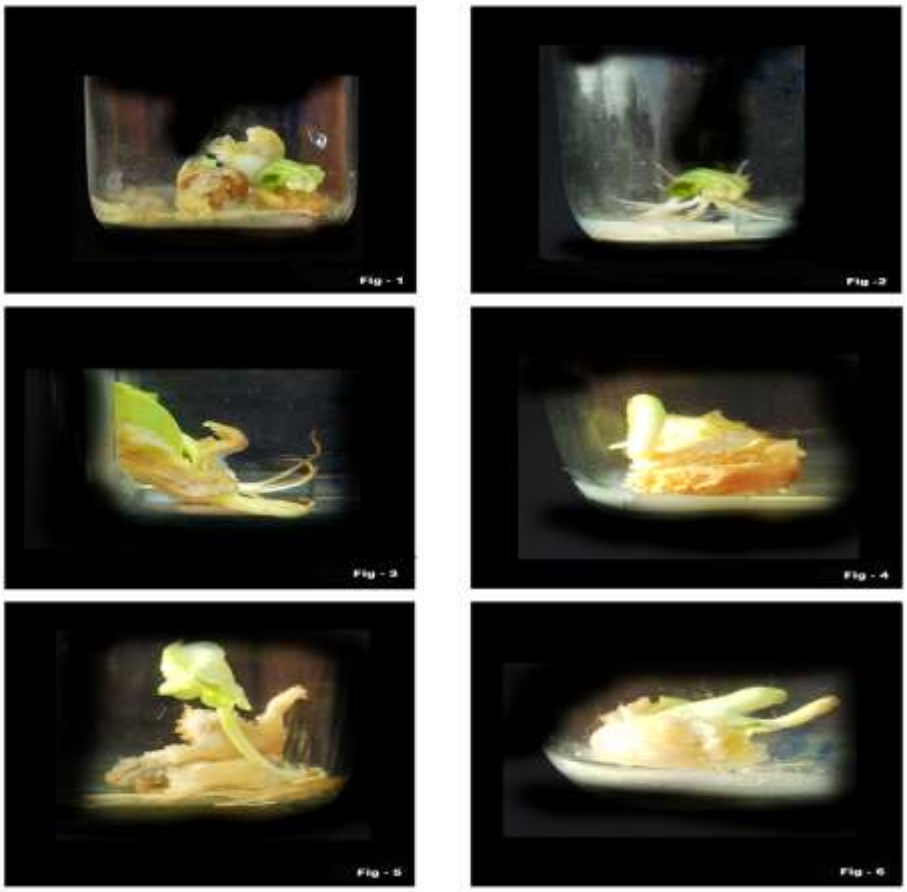


Table-4

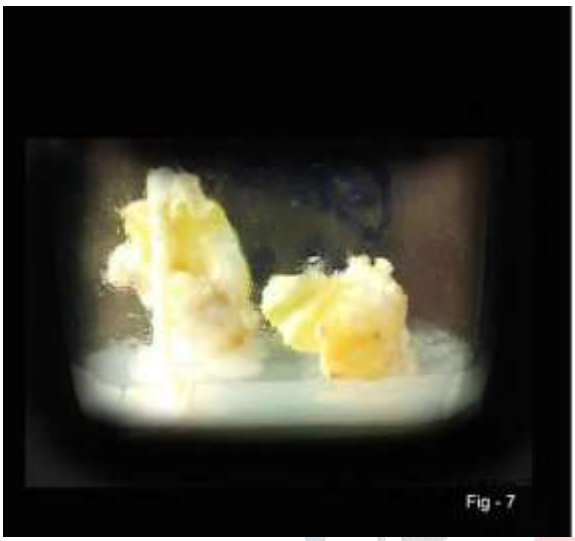
Effect of auxins and cytokinin on the embryo axis explants of *Jatropha curcas* L.

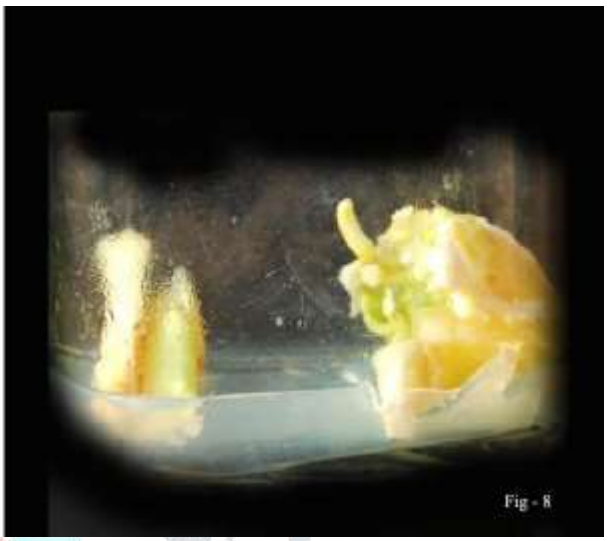
S.NO	Con. of the growth hormones (mg/l)		Morphogenetic Growth Responses		
	IAA	BAP	Callus	Shoot	Root
1.	0.5	0.5	+++	+	-
2.	0.5	1.0	++++	+	-
3.	0.5	2.0	++	+	-
4.	0.5	5.0	+	+	+
5.	1.0	0.5	+	-	+++
6.	1.0	1.0	++	-	++
7.	1.0	2.0	++	-	+
8.	1.0	5.0	+	-	-
9.	2.0	0.5	+	+	++++
10.	2.0	1.0	+	+	+++
11.	2.0	2.0	+	++	++
12.	2.0	5.0	+	++++	+
13.	5.0	0.5	+	+	++++
14.	5.0	1.0	+	++	+++
15.	5.0	2.0	+	+	++
16.	5.0	5.0	+	+	+

- - Not response ; + - 20% ; ++ - 40%
+++ - 60% ; ++++ - 80%

4.2 Effect of factorial combination of NAA and BAP

Different factorial combinations and concentrations of NAA with BAP gave various responses from the cultured embryo axis. The minimum concentrations of NAA should not gave any morphogenetic responses from the explants. 2:1 ratio of these hormone combinations was induced maximum amount of callus which was NAA 2 + BAP 1mg/l (Fig. 7). The minimum amount of callus was initiated from the node explants from nourished with NAA 0.5mg/l + all combinations of BAP. Moderate amount of callus was initiated from the combinations and concentrations of NAA with BAP which was 2mg/l of NAA with all combinations of BAP (Fig.8). This ratio was gradually reduced from the lower concentration to higher concentrations of BAP (Table-4).





Induction of plantlets

Enormous plantlets containing root and shoot were achieved through the various concentrations and combinations of NAA and BAP, which were NAA 0.5 + BAP 0.5mg/l; NAA 1 + BAP 2mg/l; NAA 1 + BAP 5mg/l. Generally shoots were four to six in numbers but roots were numerous and their length was up to 12 - 15cm. Well-developed roots were formed from the different concentrations and combinations of IAA 0.5 + BAP 0.5mg/l; IAA 1 + BAP 2mg/l and IAA 1 +BAP 5mg/l. Shoots formed on all other media, however, neither proliferate nor develop further when sub-cultured on the same media. Both shoots and roots were induced on media containing auxin and cytokinin in the ratio of 1 and more than 1. However, further development of shoots and roots did not occur on the same media after subculture (Table-4).

Table-5
Effect of auxins and cytokinin on the embryo axis explants of *Jatropha curcas* L.

S.No	Con. of the growth hormones (mg/l)		Morphogenetic Growth Responses		
	NAA	BAP	Callus	Shoot	Root
1.	0.5	0.5	+	+	+++
2.	0.5	1.0	+	-	-
3.	0.5	2.0	+	-	-
4.	0.5	5.0	+	-	-
5.	1.0	0.5	+	-	-
6.	1.0	1.0	+	+	+
7.	1.0	2.0	+	+	++++
8.	1.0	5.0	+	+	++++

9.	2.0	0.5	++++	++++	+++
10.	2.0	1.0	++++	+++	++
11.	2.0	2.0	++	++	+
12.	2.0	5.0	+	-	-
13.	5.0	0.5	++	+++	++
14.	5.0	1.0	+	++	+
15.	5.0	2.0	-	-	-
16.	5.0	5.0	-	-	-

- - Not response ; + - 20% ; ++ - 40%
+++ - 60% ; ++++ - 80%

DISCUSSION

Among different explants of *Jatropha curcas* L., embryo axis showed more growth response in culture. Young embryo axis of *J. curcas* L. has contains more meristamatic tissues than any other explants. Even though direct shoot buds were developed from embryo axis explants in culture, embryo axis are more ideal for induction of callus and multiple shoots.

Previous tissue culture work on *J. curcas* L. this laboratory (personal communication) also revealed that the embryo explants form the best source of explants for tissue culture work. Lee Stadelmann *et al*. (1989)[37] and Mroginshi *et al*. (1981)[38] also demonstrated in popular tissue culture that embryo axis explants showed higher regenerative capacity than any other organs. Embryo axis explants are more sensitive to 2,4-D when compared to IAA, IBA, NAA and BAP. Minimum concentration of 2,4-D (5 mg/l) was able to induced maximum callus when compared to IAA , NAA and IBA, among the auxins. The sensitivity of plant tissues to 2,4-D in culture is well documented in earlier works.

Out of four growth regulators used in the present study (IAA, NAA, BAP and 2,4-D). Embryo explants of *J. curcas* L. preferred 2,4-D for callusing. Callus inducing ability of the auxins on embryo explants of *J. curcus* L. was in the following order 2,4-D > BAP > IAA >NAA. The preference of specific hormone and concentration of tissue explants of various plant species for callus induction is known already[39,40,41,42,43,44,45].

Embryo axis explants of *J. curcas* L. produced maximum callusing potential in medium supplemented with 2,4-D 5 mg/l. Different factorial combination of hormones elucidated different morphogenetic potential of root explants. Generally auxin, cytokinin ratio determines differentiation in cultured tissues. Endogenous hormone level of the explants of organs alters the exogenous requirement of plant hormones. The role of growth regulators in growth and morphogenesis of plant tissues cultured in *in vitro* was known after the pioneering work of Skoog and Miller (1957)[46]. Morphogenetic potential of tissue explants is also altered by genetic and physiological age of the mother plant[47,48,49,50].

Embryo axis explants of *J. curcas* L. produced shoots directly from explants (IAA 1mg/l + BAP 0.5 mg/l and IAA 1mg/l + BAP 2mg/l). Only one cm length of shoots were proliferated from both combinations of the hormones. The tested combinations of both hormones predicted the same results in different plants, *Eclipta prostrata*[51], *Wedelia chinensis*[52].

The shoots were appeared to develop from growth centers formed in the compact and meristematic callus mass. Formation of embryoids was not observed. Shoots formed directly from explants were originated from

the cut ends, which consist of vascular cambium. Recent work by Sudhersan (1998)[53] also revealed the formation of shoot bud from cut ends of embryo of *Enicostemma axillare*. In the conventional breeding approach of *J. curcas*, most important phenotypic traits considered in the selection of accessions are seed yield, seed size and oil yield. From literature survey, it is given to understand that this is the first report wherein such massive numbers of hybrids have been produced for commercial purpose.

SUMMARY

In the present investigation, the word famous biodiesel plant *Jatropha curcas* L. embryo axis explants were selected for this study. These explants were cultured on MS medium supplemented with different concentrations and combinations of plant hormones like IAA, NAA, 2,4-D and BAP.

It was observed that embryo axis explants have maximum morphogenetic potential than other explants of *J. curcas* L. Optimum concentration of individual hormone for the growth of embryo axis explants was determined. It was found to be 5mg/l for 2,4-D and NAA; 2 mg/l for IAA and 1mg/l for BAP.

Factorial combination of different concentrations and combinations of IAA and BAP was tried to ellicit morphogenetic potential of embryo axis explants. Compact and light brown colour callus was observed on the all combinations of IAA and BAP. 0.5mg/l of IAA and 1mg/l of BAP combinations reported to have high callulogenic responses, which was 1:10. 0.5mg/l of IAA with all concentrations of BAP (0.5, 1, 2, 5mg/l) showed only callus.

Shoot induction from the embryo axis explants was observed in the combinations of IAA 1mg/l with 0.5 and 2mg/l of BAP. The shoot grew only 1cm but has not elongated.

Minimum amount of calli were induced on the media containing 2 and 5mg/l of IAA with all concentrations of BAP (0.5,1, 2 and 5mg/l).

Generally moderate amount of calli were observed on the media containing all the combination and concentrations of NAA (0.5, 1, 2 and 5mg/l) and BAP (0.5, 1, 2, 5mg/l). But only minimum callus was observed in the combination and concentrations of NAA and BAP such as 1 +0.5, 1 +1 and 5 + 5 + 0.5mg/l.

Jatropha curcas is a widely distributed crop with high level of adaptability based on the agroclimatic conditions. This has resulted in varying seed yields in different geographic locations. The floral characters and floral biology is pre-requisite to harness its yielding capabilities in order to produce high yielding varieties.

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