CHAPTER-2

Tissue Culture Studies For Development of Regeneration Protocol for *Jatropha*

2.0 Introduction

Jatropha curcas is a drought resistant shrub belonging to the genus Euphorbiaceae. It has been touted as a biodiesel plant. The first commercial application of its seed oil was reported from Lisbon where the oil was used for soap production and for lighting lamps. The press cake was used as fertilizer for potatoes (Gubitz et al, 1999).

Today, *Jatropha curcas* is mainly cultivated as a biofuel crop on marginal land for poverty alleviation. *Jatropha* has also been exploited for its medicinal properties. The seeds of this plant are used as a purgative, sap for wound healing, decoction of leaves for malarial treatment and many more.

In India it is generally planted as a fence around the fields to protect it against grazing cattle and it is also found to reduce soil erosion. In Comore islands, in Papua New Guinea and in Uganda, *Jatropha curcas* plants are used as a support for Vanilla plants and as a source of shade for Coffee plants in Cuba (Henning, 2004).

However, its commercial success as a biofuel plant is still elusive. Major bottlenecks for the same are significant variations in seed yield and oil content, low seed viability and germination rate. For vegetative propagation to be successful, it would require availability of quality planting material throughout the year. However, this is limited by unavailability of quality planting material and propagation being affected by climatic changes.

Therefore, improvement in programmes of popularizing *J. curcas* by modern methods of agro-biotechnology is of interest worldwide. This has increased the importance of developing tissue culture protocols to facilitate large scale production of true-to-type plants. These protocols could also be used for the improvement of the species by genetic engineering techniques. *In vitro*

regeneration techniques offer a powerful tool for germplasm conservation, mass multiplication of true-to-type plants and genetic transformation (Kumar and Reddy, 2010).

Genetic transformation approach allows introduction of novel genes thereby helping in introducing agronomically important traits. The availability of an efficient regeneration system is a prerequisite for utilizing this approach. For successful genetic modification, efficient production of transgenic plants, effective and fast regeneration system is imperative. Though several reports on regeneration from various explants in *J. curcas* exist, not many reports on its genetic transformation are present. This is due to the lack of efficient protocols to regenerate whole plants through *in vitro* regeneration from the transformed tissue.

2.1 Literature Studies

Few regeneration protocols have been reported for *J. curcas* using different explants like leaves (Sujatha and Mukta, 1996; Sujatha et al, 2005; Deore and Johnson 2008; Reddy et al, 2008; Khurana-Kaul et al, 2010; Divakara et al, 2010); shoot tips (Rajore and Batra, 2005); nodes and axillary nodes (Sujatha et al, 2005); petiole (Sujatha and Mukta, 1996, Kumar and Reddy, 2010, Kumar et al, 2011); hypocotyls (Sujatha and Mukta, 1996); cotyledons (Kumar et al, 2010) etc. Both direct and indirect plant regeneration systems have been reported. All the above stated reports have mentioned the use of Murashige and Skoog's (MS) medium (1962). Along with the salts of MS media, phytohormones are also added. Plant hormones do not function in isolation within plant, instead, function in relation to each other. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occurs in actively dividing tissue, and of the two what predominates is decided by the ratio of cytokinin and auxin. The ratio of hormones in the media also decides the pattern of overall growth and morphological changes. In light of this, the hormone differentials in experimental media should also have an effect on the growth and development of excised explants (Mineo, 1990). The role of specific hormones for specific stages of tissue culture is well defined. Several studies have reported the use of plant growth regulators used at various stages like shoot bud induction, elongation, rooting etc. for *J.curcas*. Apart from the kind of hormone its concentration is also critical. For *J.curcas* use of varied ranges of cytokinins like TDZ

(Thiadiazuron), BAP (Benzyl amino purine), Kn (Kinetin) and auxins like IBA (Indole-3-butyric acid), NAA (Naphthalene acetic acid), IAA (Indole-3-acetic acid) have been used. Apart from phytohormones certain media additives have also been found to play a crucial role in achieving regeneration. Sujatha and Mukta (1996) reported regeneration from various explants like hypocotyls, petiole and leaf explants with a range of cytokinins like zeatin, kinetin and N6 Benzyladenine either singly or in combination with indole-3-butyric acid (IBA). This study also compared the performance of different leaves with respect to their position in tissue culture where they reported that the third leaf was more responsive as compared to the fourth leaf. Independent of the explants type, direct adventitious shoot bud induction was recorded highest on MS medium with 2.22 µM BA and 4.9 µM IBA. Half strength MS, MS basal salts without auxin, MS in combination with IAA, NAA or IBA was used to induce rooting and 80% rooting efficiency was reported. Rajore and Batra (2005) reported shoot tip culture on MS with 8.87 µM BAP and 2.85 µM IAA. They also reported the incorporation of adenine sulphate, glutamine and activated charcoal as media additives. They reported rooting on half-MS with 2.46-24.6 µM IBA. Half strength MS in combination with 3.0 mg/l IBA favored root formation in the same study. Sujatha et al, 2005 reported shoot bud induction from axillary nodes of *Jatropha curcas* on MS with 2.3-46.5 µM Kn, 2.2-44.4 µM BA and 2.3-45.4 µM TDZ individually. Leaf segments were cultured on MS +8.9 µM BA +2.5 µM IBA. Incorporation of TDZ was found to favor shoot bud induction as compared to other cytokinins. The elongated shoots were rooted on medium comprising of half strength MS basal salts and supplemented with 5.4 µM NAA. MS medium supplemented with 22.19 µM BAP, 2.32 µM Kn and 0.57 µM IAA facilitated shoot bud induction from nodal explants of *J.curcas* (Kalimuthu et al, 2007). Effectiveness of TDZ in inducing direct organogenesis from leaf discs of *J.curcas* was first reported by Deore and Johnson (2008). Though earlier reports suggested direct organogenesis with BAP and IBA, Khurana-Kaul et al, 2010 reported intermediate callus with the use of the same combination, however, they also reported the effectiveness of TDZ along with IBA. In this study they varied $CuSO_4$ levels and found it to be more effective at ten times higher concentration than that of MS salts. This resulted in significant improvement in shoot bud induction. The role of TDZ in significantly influencing shoot bud induction in *J.curcas* was also substantiated by Kumar and

Reddy, 2010; Kumar et al, 2011. They also mentioned the orientation of the explant as an important factor for shoot bud formation.

Shoots can be derived either through differentiation of non-meristematic tissues known as adventitious shoot formation or through pre-existing meristematic tissues known as axillary shoot formation. Both the approaches require synergistic interaction of physical and chemical factors. A successful plant regeneration protocol requires appropriate choice of explant, age of the explants, definite media formulations, specific growth regulators, genotype, source of carbohydrate, gelling agent and other physical factors including light regime, temperature, humidity, etc. Genotype is one of the main factors that influence the organogenic response of cultures in different plant species. Among the different factors affecting *J.curcas* regeneration, the genotypic dependence is ranked quite high (Mukherjee et al, 2011).

From the above discussion it is evident that a need to derive a genotype dependent effective regeneration protocol is imperative and that would pave the way for further genetic transformation studies.

2.2 Materials and Methods

2.2.1 Plant Material and general culture conditions

Jatropha curcas plants grown on The M.S. University Campus were used as source of explants. Tissue culture media were prepared as per specifications in Murashige and Skoog (MS) (1962) having 30g/L sucrose. The pH of the media was adjusted to 5.6 ± 0.1 using 1N NaOH or 1N HCl and supplemented with 7g/L agar. Media was autoclaved at 15 psi at 121°C for 20 minutes. Growth conditions were maintained at 26±2°C with 16 h photoperiod with flux density of 30 µmol m^{-2} s^{-1} by cool white fluorescent tubes (Philips, India).

2.2.2 *Jatropha curcas* explants

Young leaves at 3rd and 4th node from the apex were collected from 1.5-2 year old plants grown on campus. The explants were thoroughly washed with tap water for nearly half an hour followed by washing with soap solution. The explants were surface sterilized with 0.1%

mercuric chloride for 2 to 3 minutes followed by five rinses with sterile distilled water. The detailed protocol for explant surface sterilization of *J.curcas* is presented in section 2.3.1. Leaves were then excised into small pieces of 1x1cm and inoculated on Murashige & Skoog (MS) basal medium. After a week these leaf discs were transferred to media containing different combination of BAP and IBA for regeneration. They were observed periodically and their properties were recorded. A week after inoculation on MS basal medium, the explants were cultured on (4.5 µM-27.0 µM) BAP and (3.50 µM-7.5 µM) IBA. enriched media

For experiments on culture initiation from axillary buds, tender, thin, green twigs cut below 2nd and 3rd nodes were taken as explants. Each explant would contain one apical bud and 2 to 3 axillary buds. Nodes after collection were pretreated and sterilized for 6 minutes using 0.1% mercuric chloride trimmed to 1.0-1.5 cm and cultured. The culture medium comprised of MS medium supplemented with 2.2 µM BAP and 4.9 µM IBA. These cultures were then sub-cultured on MS medium supplemented with 8.9 µM BAP and 2.9 µM IAA with adenine sulphate (100mg/l) and glutamine (100 mg/l) as an addendum. The cultures were incubated at 26 ± 2 °C under a 16 h photoperiod using cool, white fluorescent light (30 µmol m^{-2} s^{-1}).

2.2.3 Data Analysis

Experiments were set in completely randomized design. (CRBD) block

All the experiments were repeated thrice and had ten replicates with single explants. Observations on number of explants forming callus, bud initiation and number of explants forming multiple shoots per explants were recorded. All data obtained were subjected to analysis of variance and significant differences in values were calculated according to Duncan's Multiple Range Test (DMRT).

2.3 Results and Discussion

2.3.1 Standardization of surface sterilization treatment

include in material & method

Surfaces of plant parts carry a wide range of micro-organisms. Micro-organisms as common companion to plant roots have been well documented but there have been fewer reports describing their existence in the aerial parts of plants. For this reason the plant shoot tissues, especially the meristems, have widely been considered sterile. Nevertheless the vascular tissues

Where is result?

are regularly colonized by bacteria and some fungi are known to inhabit the leaves of plants (Pirttila et al, 2000). For these reasons microbial contamination is one of the most serious problems of tissue culture. This has been reviewed exhaustively by Leifert and Cassells, 2001. There are two major ways of achieving axenic cultures. The first and convenient procedure is to produce the explants under aseptic conditions, for example aseptically germinated seedlings. For the second type i.e. production of explants under non-aseptic conditions, conditions vary. The first step should be removal of dirt and debris from the plant tissue followed by washing with a detergent solution. Detergent is removed by several rinses in distilled water prior to sterilization. Contamination with microorganisms is considered to be the single most important reason for losses during *in vitro* culture of plants. During sterilization, the living plant material should not lose its biological activity and only contaminants should be eliminated; therefore explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji et al, 2009). The disinfectants widely used are sodium hypochlorite, ethanol, calcium chloride, mercuric chloride etc. Sodium hypochlorite is usually purchased as laundry bleach. Its exact mode of action is unknown however, it (NaOCl) is known to form hypochlorite (HOCl) which has bactericidal activity. It is readily available and can be diluted to proper concentrations. A balance between concentration and time must be determined empirically for each type of explant to prevent phytotoxicity. The standard protocol for these procedures can be found at our University website (https://www.msu.edu/course/css/451/).

Though ethanol is a powerful sterilizing agent, it is extremely phytotoxic. Therefore, its use in surface sterilization of plant material is restricted to a few seconds or minutes. Its use also depends upon the nature of plant material for example very young and tender tissue like young leaves, young petioles etc. are given a very short treatment. Generally 70% ethanol is used prior to treatment with other compounds.

To enhance effectiveness in sterilization procedure, a surfactant like Tween 20® is frequently added to the sterilizing solution (and in some laboratories mild vacuum is applied during the procedure); in general, the sterilizing solutions containing the explants are continuously stirred during the sterilization period (https://www. msu.edu/course/css/451/).

Mercuric chloride is extremely toxic to plants and humans and must be disposed off with care. Since mercury is phytotoxic, it is critical that many rinses be used to remove all traces of the mineral from the plant material. In a nutshell, to enhance the effectiveness of sterilization procedure surfactant (e.g. Tween 20) may be frequently added to the sodium hypochlorite, a mild vacuum may be used during the procedure and the explants are often shaken or continuously stirred (https://www. msu.edu/course/css/451/). A successful tissue culture protocol starts with effective explant sterilization (Dodds and Roberts, 1985).

The aim of the present investigation was to find out a suitable surface sterilization protocol that would ensure maximum explants survival with minimum contamination and also be cost effective and time efficient. Surface sterilization was followed as per standard procedure with 0.1 % mercuric chloride with different time duration for nodes and leaves, however they turned brown and no growth was observed in the cultures. Reducing the concentration and/or duration of exposure resulted in heavy fungal contamination. Initially variation in pre-treatment and mercuric chloride time duration was done to study its effect on achieving axenic cultures. Leaf explants dried and finally died owing to longer duration of exposure to mercuric chloride. Plant species with rich secondary metabolites such as *Jatropha curcas*; have proved to be difficult for mass propagation through tissue culture (Roy, 1995).

As shown in table 2.1, APSA (all purpose surface adjuvant) was used as a surfactant. In the pre-treatment virosyl (10%) was used to enhance the process of surface sterilization. However, no improvement was seen in survival of explants. It has been reported that the use of field grown plants as a direct source of explant material for the production of 'clean' *in vitro* plantlets presents a major challenge (Webster et al, 2003).

Results reported in table 2.1 shows that a combination of APSA, labwash, Bavistin, streptocyclin and virosyl with 0.1% mercuric chloride for 6 - 10 min did not yield good results. So in the next set of experiments NaOCl was added as pretreatment II and Bavistin, streptocyclin and ciprofloxacin were used in pretreatment III (table 2.2). The time of pretreatment to mercuric chloride was decreased to 1 min for leaves and 6 min for nodes.

Table 2.1: Explant survival and contamination with varied pretreatment conditions

Pretreatment I	Pretreatment II	Pretreatment III	Sterilant Concentration and duration of treatment	Number of Explants inoculated	Number of explants contaminated
Labwash +APSA	---	IPA (70%) (1 minute)	0.1% $HgCl_2$ (10minutes)	30 (N) 30 (L)	30 (N) 30 (L)
Running tap water (30 minutes) ↓ Labwash +APSA	---	IPA (70%) (1 minute)	0.1% $HgCl_2$ (8 minutes)	30 (N) 30 (L)	30 (N) 30 (L)
Labwash +APSA ↓ Bavistin+ Streptocyclin (O/N)	APSA+ Vacuum Infiltration	---	0.1% $HgCl_2$ 8 minutes-N 6 minutes-L	30 (N) 30 (L)	30 (N) 30 (L)
Labwash +APSA ↓ Virosyl (10%) (30 minutes) ↓ Bavistin + Streptocyclin (O/N)	APSA+ Vacuum Infiltration	---	0.1% $HgCl_2$ 8 minutes-N 6 minutes-L	30 (N) 30 (L)	23 (N) 30 (L)

Key: Nodes (N), Leaves (L), Overnight (O/N), All Purpose Surface Adjuvant (APSA), Isopropanol (IPA)

Variation in time duration of mercuric chloride as well as sodium hypochlorite in pre-treatment were tried to obtain sterile node and leaf explants. NaOCl was incorporated in the pre- treatment step to reduce fungal contamination. Time of exposure to $HgCl_2$ was also varied.

Table 2.2: Explant survival and contamination with NaOCl in pretreatment and varying $HgCl_2$ duration

Pretreatment I	**Pretreatment II**	**Pretreatment III**	**Sterilant concentration and duration of treatment**	**Number of Explants inoculated**	**Number of explants contaminated**
Running tap water ↓ Labwash + APSA	3% Sodium Hypochlorite (7 minutes)	IPA (70%) (1 minute)	0.1% $HgCl_2$ 8 minutes-N 6 minutes-L	30 (N) 30 (L)	15 (N) 30 (L)
Running tap water ↓ Labwash + APSA	3% Sodium Hypochlorite (6 minutes)	IPA (70%) (1 minute) ↓ Bavistin + Streptocyclin (2 hours)	0.1% $HgCl_2$ 6 minutes-N 5 minutes-L	30 (N) 30 (L)	19 (N) 10 (L)

Pretreatment I	**Pretreatment II**	**Pretreatment III**	**Sterilant concentration and duration of treatment**	**Number of Explants inoculated**	**Number of explants contaminated**
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 2 minutes-L	IPA (70%) (I minute) ↓ Bavistin+ streptocyclin (2 hours)	0.1% $HgCl_2$ 7 minutes-N 1 minute-L	50 (N) 30 (L)	25 (N) 30 (L)
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 2 minutes-L	Bavistin+ streptocyclin (O/N) ↓ IPA (70%) (I minute)	0.1% $HgCl_2$ 6 minutes-N 1 minute-L	45 (N) 30 (L)	45 (N) 30 (L)

Pretreatment I	**Pretreatment II**	**Pretreatment III**	**Sterilant concentration and duration of treatment**	**Number of Explants inoculated**	**Number of explants contaminated**
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 1 minute-L	Bavistin+ ciprofloxacin (2 hours) ↓ IPA (70%) (I minute)	0.1% $HgCl_2$ 6 minutes-N 1 minute-L	40 (N) 70 (L)	31 (N) 70 (L)
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 1 minute-L	Bavistin+ ciprofloxacin (1 hours) ↓ IPA (70%) (I minute)	0.1% HgCl2 6 minutes-N 30seconds-L	40 (N) 60 (L)	30 (N) 36 (L)

However, both NaOCl and mercuric chloride were proving detrimental to the survival of explants. Hence, NaOCl was removed from the pre-treatment step and Bavistin, a systemic fungicide was included as a pre-treatment and its treatment time was standardized to 2 hours. The use of antibiotics in certain growing cultures was found to be inhibitory. Also $HgCl_2$ treatment duration was modified and survival of axenic cultures increased. Webster et al, 2003 cited Barrett & Cassells, 1994 stating that uninformed use of antimicrobial chemicals (such as antibiotics) may cause phytotoxicity, retard explants growth and encourage the buildup of resistance. Furthermore, even though some antibiotics may give comparatively high activity

when tested on defined bacteriological media, these results are not usually replicable on the complex tissue culture media and so the expected results are usually elusive.

Table 2.3: Explants survival and contamination with varying time of exposure to fungicide and $HgCl_2$

Pretreatment I	**Pretreatment II**	**Pretreatment III**	**Sterilant concentration and duration of treatment**	**Number of explants inoculated**	**Number of explants contaminated**
Running tap water ↓ Labwash + APSA	Bavistin+ Streptocyclin (O/N)	IPA (70%) (1 minute)	0.1% $HgCl_2$ 7 minutes-N 1 minute-L	30 (N) 30 (L)	30 (N) 29 (L)
Running tap water ↓ Labwash + APSA	Bavistin (1.5 hours)	IPA (70%) 1minute- (N) 30 seconds-(L)	0.1% $HgCl_2$ 3 minutes-N 1 minute-L	125 (N) 43 (L)	96 (N) 2 (L)

Pretreatment I	**Pretreatment II**	**Pretreatment III**	**Sterilant concentration and duration of treatment**	**Number of explants inoculated**	**Number of explants contaminated**
Running tap water ↓ Labwash + APSA	Bavistin (2 hours)	IPA(70%) 1minute-(N) 30 seconds-(L)	0.1% $HgCl_2$ 3minutes-N 1 minute-L	75 (N)	9 (N)
Running tap water ↓ Labwash + APSA	Bavistin (2 hours)	IPA(70%) 1 minute-(N) 30 seconds-(L)	0.1% $HgCl_2$ 3minutes-N 1 minute-L	82 (N)	12 (N)

Explant contamination is a function of several plant and environmental related factors such as plant species, age, explant source and prevailing weather condition. Despite the best timing and selection efforts it is almost impossible to eliminate contamination from *in vitro* grown plants (Webster et al, 2003). From the above results it was concluded that after rinsing the explants in running tap water, they should be washed with detergent followed by Bavistin treatment for 2 hours. Once these pretreatments were over, explants were treated with 70% IPA for one minute

to 30 seconds for nodes and leaves respectively. Surface sterilization was done using 0.1% $HgCl_2$for 4 minutes for nodes and 1 minute for leaves.

2.3.2 Indirect organogenesis through leaf discs

The regeneration of plants through an intermediate callus phase is called "indirect regeneration." The explants (meristematic tissue) dedifferentiate into callus, an unorganized growth of dedifferentiated cells. Group of cells in callus reorganize to form meristemoid, similar to meristem tissue. This then redifferentiates to form shoot buds, which finally regenerates to plantlets. In this study, adventitious shoot regeneration from leaf explants was attempted.

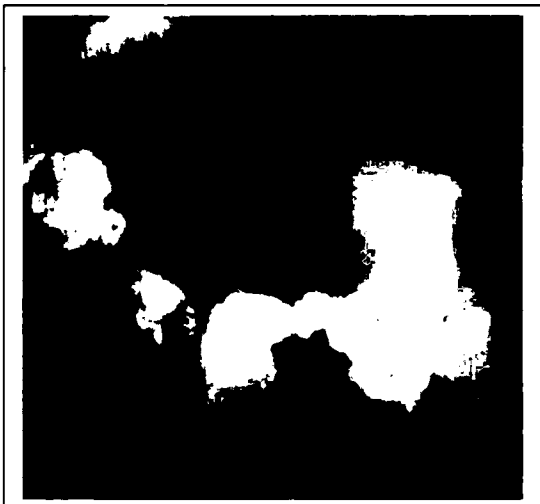

Figure 2.1: Callus initiation at the cut margin

It is a known fact that a balance between auxin and cytokinin normally induces effective organogenesis. Though the nature of interaction between the two plant growth regulators is not completely understood, cell division seems to be regulated by their interactions affecting different phases of cell cycle. While auxins are known to exert an effect on DNA replication, cytokinin exerts some control over the events leading to mitosis (Pasternak et al, 2000). Therefore normal cell divisions would require synchrony between S phase and cell division suggesting that auxin and cytokinin levels in culture be carefully matched. Reports have shown the importance of BAP and IBA in inducing organogenesis from leaf discs (Sujatha and Mukta, 1996; Rajore and Batra, 2005; Sujatha et al, 2005). In the current investigation a range of combination of BAP (4.5 µM - 27.0 µM) and IBA (3.0 µM -7.5 µM) were used for indirect organogenesis. Curling of enlarged leaf discs was first observed followed by callus appearance at the cut margins (figure 2.1).

No significant difference was observed in callusing and regeneration frequency from the explants derived from 3rd and 4th expanding leaf. This is in contrast to the result reported by Sujatha and Mukta, 1996 wherein 50% regeneration was seen in leaf discs obtained from 3rd expanding leaf and 30% from the 4th expanding leaf.

Figure 2.2: Emerging shoot buds from callus

Callus morphology and relative response of callusing was studied. It was observed that in MS media supplemented with both BAP and IBA at highest levels i.e. 27.0 µM and 7.5 µM respectively, callus formation was very less and much slower as compared to other combinations. BAP levels as low as 9.0 µM with 5.0 µM IBA also failed to induce sufficient callus (table 2.4).

Table 2.4: Various media combinations studied for callus formation in leaf explants of *J.curcas*

BAP/IBA (µM)	Total No of explants	Number of explants showing callus	Callus Morphology within a week/ after week	Presence of Callus	Time of Callus appearance	Relative response to Callus formation
4.5/3.0(B)	30	27	White/Green + White	Over entire margin	Within two weeks time	++
13.0/3.0(C)	30	30	White/Green + White	Over entire margin + bulb of Callus at edge	Immediately after a week	+++
27.0/3.0(D)	20	18	White/Green + White	Over entire margin + bulb of Callus at edge + small calli over midrib	Initial fast Development of Callus followed by slow development	++

BAP/IBA (μM)	**Total No of explants**	**Number of explants showing callus**	**Callus Morphology within a week/ after week**	**Presence of Callus**	**Time of Callus appearance**	**Relative response to Callus formation**
13.0/5.0(F)	05	18	White/Green + White	Over the surface	Initially slow then fast	+++
27.0/5.0(G)	05	29	Green + White	Over entire leaf margin	Appeared after two weeks	+++
4.5/7.5(H)	04	16	Green + White	On one entire side	Appeared after two weeks	+++
27.0/7.5(J)	06	24	Green + White	On one side	Appeared after two weeks or so	+

Explants on MS medium supplemented with lower levels of IBA (3.0μM and 5.0 μM) and higher levels of BAP gave maximum callus formation. Similar results were obtained in MS fortified with high levels of IBA in combination with low BAP. Though MS media containing highest levels of either BAP or IBA showed massive callus formation no organogenesis was observed. Even on sub-culturing on respective media it continued to form callus. In the medium

supplemented with 13.0 µM BAP + 7.5 µM IBA and 27.0 µM BAP + 7.5 µM IBA, callus turned brown and dried after first passage. By reducing IBA to 3.0 µM in combination with all three concentrations of BAP, organogenesis was observed after 45 days in culture. In the present study, tweaking cytokinin levels did not have an effect on organogenesis while low IBA levels did influence organogenesis. Low levels of IBA play a key role in callus formation, bud initiation and multiple shoot formation. This is also substantiated by Kalimuthu et al, 2007 wherein it has been reported that high concentrations of auxins are generally inhibitory to morphogenesis, and the use of an appropriate auxin-cytokinin ratio is essential to obtain proper shoots and root primordia. This could be suggestive of the importance of lower levels of IBA in inducing organogenesis. Buds in clusters (3-5) started emerging from various locations of the callus, mostly underneath the explants as shown above in figure 2.2. First bud initiation was observed in MS medium supplemented with 27.0 µM BAP + 3.0 µM IBA. It was also the most effective combination for shoot bud initiation and proliferation (table-2.5, figure 2.3).

Table 2.5: Effect of BAP and IBA on formation of buds

BAP/IBA (µM)	Number of buds per explants
	After 6 weeks
27.0/3.0	10 ± 2.35^{a}
13.0/3.0	8.0 ± 1.41^{a}
4.5/3.0	5.0 ± 0.94^{b}
27.0/5.0	2.0 ± 0.94^{c}
4.5/7.5	$0.0 \pm 0.0^{c,d}$

Means in each column followed by same letters are not significantly different according to DMRT at α=0.05 at 12 degrees of freedom.

Figure 2.3: Shoot bud initiation and proliferation

In the current study, MS+27.0 μM BAP + 3.0 μM IBA resulted in 10 buds per regenerating calli. Out of these 9.33 buds showed proliferation on the same combination. Khurana-Kaul et al, 2010 reported 8.6 buds in MS+13.33 μM BAP + 2.46 μM IBA. Using similar combination in the present study also gave rise to 8.0 buds per regenerating calli. In the study by Khurana-Kaul et al, 2010 they have also shown the influence of TDZ in achieving higher number of shoot buds. While in the present study results have been achieved even in the absence of TDZ. Sujatha and Mukta, 1996 reported 2.22 μM BAP and 2.46 μM IBA to give 10.7 shoots per regenerating calli. A few other media combinations in the present study showed the same response a little later. As shown in table-2.6, there is a significant difference between all the media combinations studied except for the two containing isomolar concentration of IBA (3.0 μM) with varying BAP levels (13.0 μM and 27.0 μM). This indicates that the increased level of cytokinin fails to show its impact on initiation of organogenesis. However, after organogeneses have been successfully induced and shoot bud proliferation is observed increased IBA levels have little influence (table-2.6).

Table 2.6: Effect of BAP and IBA on proliferation of buds

BAP/IBA (μM)	Number of buds per explants
	After 10 weeks
27.0/3.0	9.33 ± 0.27^{a}
27.0/5.0	8.66 ± 0.54^{a}
13.0/3.0	6.00 ± 1.63^{b}
4.5/3.0	5.33 ± 1.90^{c}
4.5/7.5	0.0 ± 0.0^{d}

Means in each column followed by same letters are not significantly different according to DMRT at α=0.05 at 12 degrees of freedom

Of the four media combinations used in the study for formation of new buds, MS medium supplemented with 13.0 µM BAP +3.0 µM IBA did not show further response. However, other media combinations continued to show their effect on the number of shoot regenerants. Table-2.7 depicts the mean length of regenerants with shoot length of more than 1.0 cm. In some explants, regenerants of more than 1.5-2.0 cm were obtained (figure 2.4).

Table 2.7: Effect of BAP and IBA on shoot length of bud regenerants

BAP/IBA (µM)	Mean shoot length	% frequency
4.5/3.0	>1.0cm	62.5
27.0/3.0	>1.0cm	55.5
27.0/5.0	>1.0cm	0

Shoot length of regenerants measuring more than 1.0 cm only have been considered.

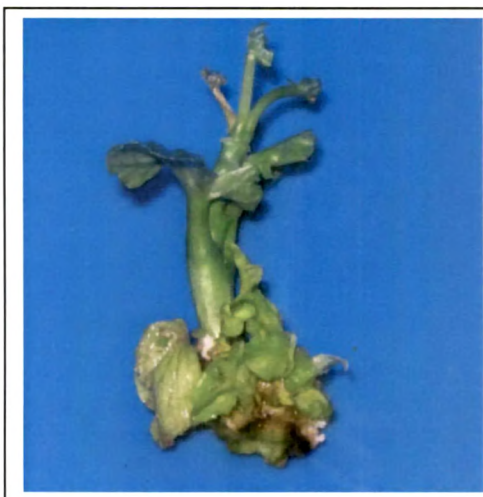

Figure 2.4: Regenerants achieved from callus

Number of shoots per explant is presented in table-2.6. The MS medium supplemented with 4.5 µM BAP + 3.0 µM IBA and 27.0 µM BAP + 3.0 µM IBA are not significantly different according to DMRT (α=0.05). Kumar et al, 2010 achieved elongation on MS+ 4.5 µM BAP + 7.5 µM IBA with shoot length of 2.3-2.5cm.

The current investigation reports an effective combination for successful regeneration of shoots from leaf discs derived calli. It could be concluded that auxins play a greater role than cytokinins in organogenesis of *J.curcas.*

While wide changes in cytokinin levels did not yield significant changes in shoot bud induction, a small increment in auxin levels gave rise to appreciable changes in the same. Hence, it could be concluded that irrespective of BAP levels, low levels of IBA are essential in order to obtain multiple shoot regenerants per explant. Deore and Johnson (2008) have shown the synergistic role of thiadiazuron (TDZ) and BA in inducing shoots. They have also mentioned about reduced effect of BA in absence of TDZ. In the present study MS supplemented with 4.5 µM BAP + 3.0 µM IBA and 27.0 µM BAP + 3.0 µM IBA showed 100% frequency for callus formation, shoot bud induction and proliferation. Shoot elongation was studied in MS+8.8 µM BAP+ 2.5 µM IAA, MS+8.8 µM BAP+ 2.5 µM IAA+1.0 µM Kn, MS+22.0 µM BAP+ 2.5 µM IAA+1.0 µM Kn and MS+8.8 µM BAP+ 2.5 µM IAA+5.0 µM Kn. Out of these combinations studied, sprouting of new shoots was observed in MS+8.8 µM BAP+2.5 µM IAA+1.0 µM Kn and MS+22.0 µM BAP+ 2.5 µM IAA+1.0 µM Kn. MS+8.8 µM BAP+ 2.5 µM IAA+1.0 µM Kn also favored quick and effective growth of existing shoots along with MS+8.8 µM BAP+ 2.5 µM IAA+5.0 µM Kn. It is a widely known fact that root formation in *in vitro* grown plants is triggered or induced by external application of auxins. In the work reported on *J. curcas* MS in conjunction with NAA (Sujatha eta al, 2005) and IBA (Deore and Johnson, 2008) respectively has been used for root formation. In the present study, rooting was observed after three weeks of transfer in the rooting medium (figure 2.5).

Various media combinations were studied to induce rooting in regenerated shoots that had achieved length of more than 1.5-2.0 cm. The media combinations used to study rooting were MSB +11.0 µM NAA, MSB +21.0 µM NAA, MSB +50.0 µM NAA, Half strength MS+9.8 µM IBA+2.22 µM BAP, MS basal medium and direct transfer of the explants to vermiculite. Rooting could be achieved on MS basal medium. This is in contrast to the studies reported by Sujatha and Mukta, 1996 wherein rooting was observed on MS basal medium within 8-10 days with 88% rooting frequency. However, in the present investigation, more time was taken for root formation.

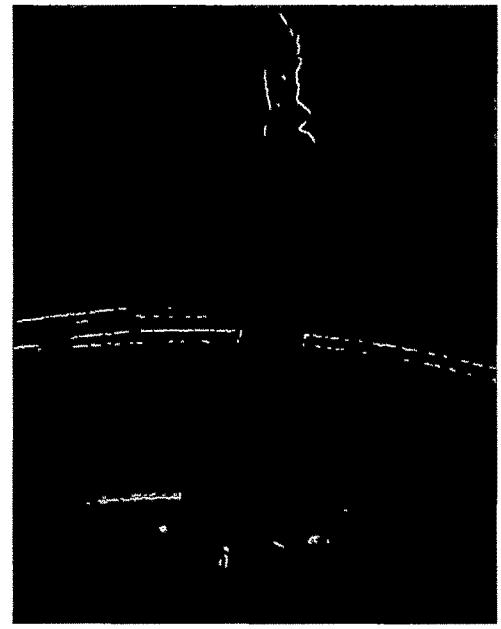

Figure 2.5: Root formation

put reference by increased year order

2.3.3 Direct organogenesis in *J.curcas*

Direct shoot regeneration has been reported from a number of explants like shoot tip, axillary node, petiole, leaf disc, immature embryo, cotyledonary leaf, epicotyls, hypocotyls etc. of toxic and non-toxic varieties of *J.curcas* (Rajore and Batra, 2005; Sujatha et al, 2005; Kalimuthu et al, 2007; Deore and Johnson, 2008; Kumar and Reddy, 2010; Shrivastava and Banerjee, 2008; Kumar et al, 2010a, b). Of all these reports, only a few have reported direct organogenesis from shoot tips, nodes or axillary buds as explants (Rajore and Batra, 2005; Sujatha et al, 2005; Kalimuthu et al, 2007; Shrivastava and Banerjee, 2008). Direct plant regeneration without an intervening callus phase is a more reliable method for multiplication or clonal propagation. The plants produced by direct organogenensis may exhibit greater genetic stability than those produced via callus mediated organogenesis (Mukherjee et al, 2011). Murashige and Skoog's medium is the reported media for all the above mentioned studies on *J.curcas*. Plant growth regulators used for direct regeneration include TDZ, BA, Kn, IBA, IAA. These growth regulators were used at different levels either singly or in combination.

In the present study for experiments on culture initiation from axillary buds, tender, thin, green twigs cut below 2nd and 3rd nodes were taken as explants. Each explant containing one apical bud and 2 to 3 axillary buds were pretreated and surface sterilized with mercuric chloride. The culture medium comprised of MS medium supplemented with 2.2 µM BAP and 4.9 µM IBA. Nodal explants produced single shoots within 10-12 days (figure 2.6). Similar results have been reported by Kalimuthu et al, 2007 where MS basal medium was used for culture initiation. Further multiplication of shoots was achieved in MS fortified with BAP, Kn and IAA. Sujatha et al, 2005 reported the use of BA, Kn and TDZ. Except for one concentration of kinetin, none of the combination with the same showed any visible signs of explants differentiation except for bud emergence with very low axillary proliferation. It is also reported here that TDZ was more effective as compared to BA and Kinetin. It is a common fact that cytokinins and auxins were found essential for shoot multiplication. The higher concentration of auxins is generally inhibitory to morphogenesis and substitution of these reagents with an appropriate auxin-cytokinin ratio is essential to obtain proper shoot and root primordia (Kalimuthu et al, 2007). In the current investigation, explants were sub cultured on MS medium supplemented with 2.22 µM BAP and 4.92 µM IBA. Though apical bud is mostly the preferred explant for obtaining direct regeneration owing to its meristematic nature, it was found that apical bud was more prone to contamination. Multiple shoot formation was observed within a period of 30-40 days (figure 2.7). Multiplication of shoots was obtained in the same combination.

(A)

Figure 2.6: Emergence of new shoot bud

(B)

Figure 2.7: Multiplication of nodal culture

The media combinations used to study rooting were MSB +11.0 µM NAA, MSB +21.0 µM NAA, MSB +50.0 µM NAA, Half strength MS+9.8 µMIBA+2.22 µM BAP, MS basal medium and direct transfer of the explants to vermiculite. The shoots were then kept on MS basal medium for rooting. Roots were observed after nearly three weeks in MS basal medium.

From the above studies it can be concluded that MS salts fortified with 2.2 µM BAP and 4.9 µM IBA successfully gave rise to new shoots and their multiplication, ~~of shoots was also observed in~~ the ~~same.~~

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