CHAPTER-3

Study of Somatic Embryogenesis in *Jatropha curcas*: Liquid Suspension Culture and Solid Media Studies

3.0 Introduction

Jatropha curcas is a perennial shrub of the family Euphorbiaceae. It has gained importance due to the oil present in its seeds. The seed oil upon a simple esterification reaction can be converted to biodiesel. Thus, *Jatropha curcas* finds a place as a promising biofuel crop. However the average biodiesel yield per hectare is too low to make it economically viable. One of the reasons for this low yield is unavailability of quality planting material. Micropropagation is used to obtain multiple shoot culture of quality healthy plants. Multiple shoot culture technique involves a large number of steps making the procedure labor intensive and time consuming. Somatic embryos, on the other hand, have the capability to give rise to complete plantlets. Somatic embryogenesis (SE) can be initiated by two mechanisms: directly on explanted tissues, where plants are genetically identical (clonation), and indirectly (from unorganized tissues - callus). Embryo development follows a few discrete steps where pro-embryos develop via stages like globular stage, heart stage and a torpedo stage.

Various factors affect somatic embryogenesis. Role of carbon sources in regulation is discussed by Al-Khateeb, 2008. Level of endogenous hormones is considered to be one of the crucial factors determining embryogenic potential of explants (Feher et al, 2003). Auxin is one of the most important hormones in regulating SE in vitro (Cooke et al, 1993). Once embryogenesis has been induced, endogenous auxin synthesis begins via an alternate pathway. Several studies have revealed the importance of proper polar auxin transport as a prerequisite for normal morphogenesis beyond the globular stage. The removal or decrease in auxin acts as a trigger for somatic embryo development as it results in the inactivation of a number of genes enabling the embryogenesis program to proceed. This has been substantiated by the observations of Halperin and Wetherell, 1964; Borkird et al, 1986. Embryogenic cultures (E) have higher contents of

endogenous auxins than their Non- Embryogenic (NE) counterparts (Jimenez and Bangerth, 2001, Rajasekaran et al, 1987). However, there are a few reports in which no differences could be found between Embryogenic and NE cultures (Michalczuk et al, 1992).

3.1 Literature Studies

Regardless of whether the adult plant is an ephemeral weed or a long-lived sequoia tree, its developmental origin is essentially the same: a seed containing a simple mature embryo that displays an apical–basal axis of polarity and a radial pattern of concentric tissue layers perpendicular to the apical–basal axis. The axis of polarity has, at its top end, the primary shoot meristem, which is flanked by one or two cotyledons, and the primary root meristem at its bottom end. The basic body organization is influenced by potentially six different genomes namely, diploid zygote, triploid endosperm, diploid maternal tissue, haploid egg cell, diploid central cell and haploid sperm cells. During embryo development, the body axes and the basic body plan of the plant are laid down. This starts with establishment of the apical–basal axis, followed by establishment of the radial axis, and finally establishment of bilateral symmetry. Importantly, the root and shoot stem cell pools, which are essential for the quasi indefinite post-embryonic growth, are also specified during this early phase of pattern formation and morphogenesis. During the subsequent maturation phase, storage reserves accumulate and ultimately the embryo prepares for developmental arrest (De Smet et al, 2010). This ability to produce morphologically and developmentally normal embryos and, indeed, whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis, resides uniquely within the plant kingdom. Since the initial description of somatic embryo production from carrot callus cells more than 35 years ago (Steward et al, 1958), this unique developmental potential has been recognized both as an important pathway for the regeneration of plants from cell culture systems and as a potential model for studying early regulatory and morphogenetic events in plant embryogenesis. Somatic embryos are induced from cultured callus cells by a relatively simple manipulation of the culturing conditions, as summarized in figure3.1. In carrot, this generally involves (1) the establishment of a callus cell line, (2) the selection of an embryogenic subpopulation of the cultured cells through sieving or gradient fractionation, (3) the removal of auxin from the culture medium, and (4) the dilution of the cells to a relatively low

density. The overall embryogenic potential of a culture is highest when the culture is relatively young (i.e., within the first year of its life) and resides primarily within a subpopulation of the culture that has been termed proembryogenic masses" or PEMs by Halperin, 1966 (Zimmerman, 1993).

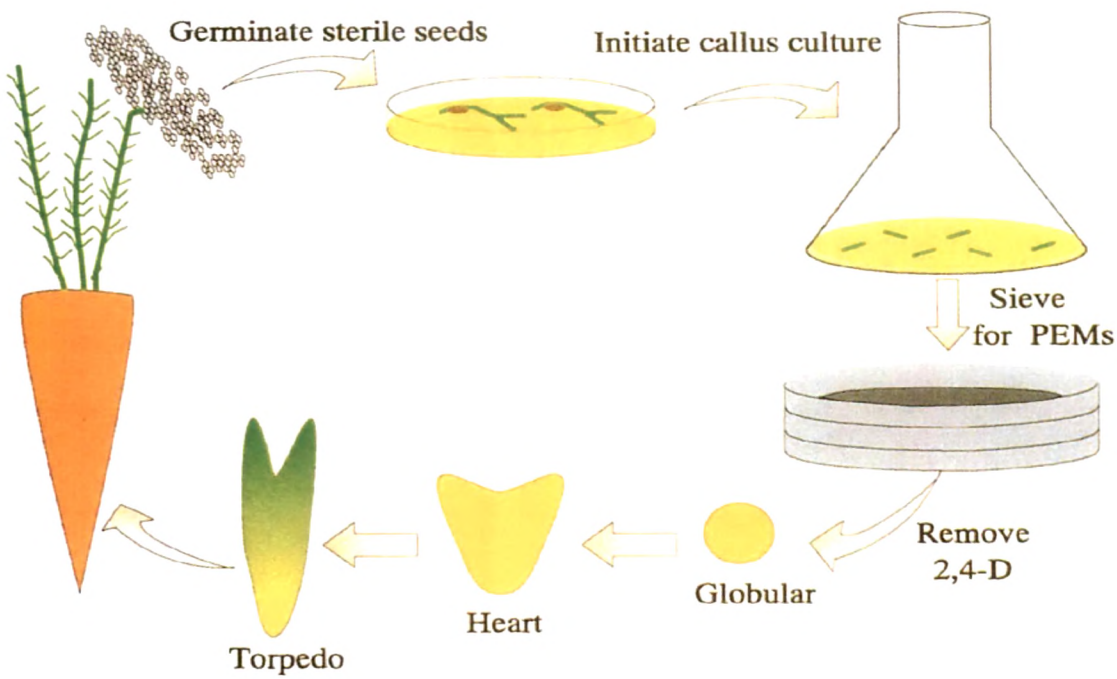

Figure 3.1: Summary of culturing of carrot somatic embryos (Zimmerman, 1993)

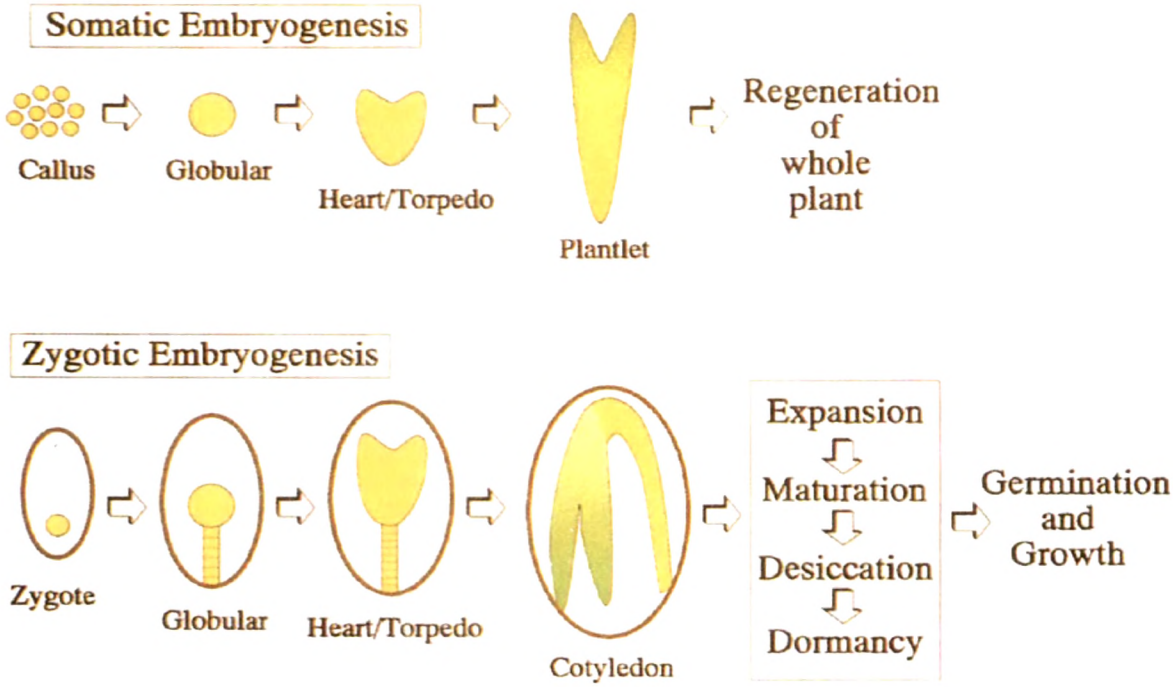

Figure 3.2: A comparison of somatic and zygotic embryogenesis (Zimmerman,1993)

Somatic embryogenesis in cultured cells is a useful system for research on differentiation to a whole plant from a single cell. Distinct phases can be physiologically distinguished in the differentiation of embryos from single cells (Nomura and Komamine, 1985). The suspension liquid culture system allows the study of different physiological and biochemical characteristics such as growth parameters, protein and DNA synthesis, plant growth regulators effect, nutrient uptake and maturation capacity, somatic embryo morphology and extracellular proteins (Salaj et al, 2007). Recently suspension cultures have also been involved in genetic transformation studies (Wenck et al, 1999).

The first phase of somatic embryogenesis is the process in which single cells divide to form embryogenic cell clusters. Exogenous auxin is required for the progression of this phase which can develop to embryos when they are transferred to the embryo-inducing medium without any trigger. This fact suggests that determination for embryogenesis occurs in the first phase. The second phase is the process in which the embryogenic cell clusters formed in the first phase develop to embryos. Development of embryos in this phase is strongly inhibited by exogenous

auxin (Nomura and Komamine, 1985). Various factors are known to influence somatic embryogenesis which includes age of the explant, storage of the explant, season of obtaining tissue from the plant source, role of auxins etc.

There are only a few reports on somatic embryogenesis in *J.curcas* (Jha et al, 2007; Kalimuthu et al, 2007). Jha et al, 2007 have reported that the type and concentration of the plant growth regulators were the strong determining factors for induction of somatic embryogenesis in *J.curcas*. Nodular embryogenic calli was observed on MS+9.3μM Kn. However, highest frequency of globular embryos was observed in the combination of 2.3μM Kn and 1.0μM IBA. Kalimuthu et al, 2007 mentioned the induction of somatic embryo on medium containing 8.87 μM kn however, further development of the embryo has not been mentioned. Soomro and Memon (2007) reported callus and cell suspension culture of *Jatropha curcas*. In the present study a systematic effort has been made to establish a protocol for somatic embryogenesis in *J.curcas* and understand the role of auxins in it. A protocol for liquid suspension culture system for *Jatropha curcas* has also been developed here.

3.2 Materials and Method

3.2.1 Plant Material

Seeds from mature and immature fruits of *Jatropha curcas* plants (1.5-2 years old) were collected and their cotyledons used as explants. Seeds were kept under running tap water for 30 minutes followed by wash with a mild detergent. They were again kept under running tap water for 30 minutes. They were treated with 70% ethanol for 30 seconds followed by three rinses with distilled water. Surface sterilization was done with 0.1% mercuric chloride for 2 minutes. Traces of mercuric chloride were removed by giving five rinses with sterile distilled water. Surface sterilized seeds were de-coated under aseptic conditions and inoculated on the respective medium.

3.2.2 Culture Media

Murashige and Skoogs (MS) salts with 0.4 mg/l, 0.8 mg/l and 1.2 mg/l 2, 4-Dichlorpphenoxyacetic acid (2, 4-D) was used for inducing callus for suspension culture studies.

MS salts supplemented with various concentration of 2,4-D (2.4 mg/l, 3.6mg/l and 4.0 mg/l and 0.4 mg/l+2% coconut water (CW), 0.8 mg/l+2% CW and 1.2 mg/l+2% CW and 0.4 mg/l+10% CW, 0.8 mg/l+10% CW and 1.2 mg/l+10% CW) and NAA (5mg/l,10mg/l and 15mg/l) were used for callus initiation and for direct somatic embryogenesis studies. The medium was fortified with 3% sucrose and agar (0.8%) was used as a solidifying agent wherever required. The pH of the media was adjusted to 5.8 before addition of agar and was autoclaved at 121°C for 20 min. Cultures were maintained at 24±2°C under 14 hour photoperiod.

3.2.3 Optimizing liquid suspension culture system

The friable callus formed was transferred to MS liquid medium and kept on shaker at 110 rpm at 25°C. The cells were harvested at regular interval of 3 days and cell counting was done. The following protocol was followed for staining and counting of cells:

3.2.4 Staining of cells in suspension cultures

1 mL of cells from suspension culture was taken asceptically in a sterile tube and centrifuged at 5000 rpm for 10 min. The cells from the pellet were stained with Evan's blue dye and observed under 40 X objective of light microscope.

3.2.5 Cell count

The number of cells per mL were counted using a hemocytometer

3.2.6 Auxin extraction and estimation

Auxin was determined by Gordon and Weber, 1951. Tissue of specific stages (0.5gm) was ground in a mortar with pestle, together with 0.01M EDTA and 0.02M Sodium diethyldithiocarbamide in 80% ethanol. This was transferred to test tube, kept on ice bath for 15-20 minutes with intermittent shaking (Sinha and Basu, 1981). This mixture was centrifuged at 5,000 g for 20 minutes at 4 °C. Auxin was estimated in the supernatant at 530 nm.

3.3 Results and Discussion

Somatic embryogenesis acts as a powerful tool for genetic improvement of any plant species because of its single cell origin. Somatic embryogenesis offers promise for rapid multiplication and genetic transformation of plants (Finer, 1988). Somatic embryogenesis can be initiated by two mechanisms: directly on explanted tissue where plants are genetically identical (clonation) and indirectly from unorganized tissues (callus). In the current investigation we report both direct and indirect somatic embryogenesis. The experiments were carried out on solidified media as well as liquid medium for suspension cultures. The advantage of liquid suspension culture system is that the growth of plant cells is more rapid than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed. In the current study the roles of a few factors that have an influence on the performance of explants in culture conditions have been discussed. Age of seeds is known to be an important parameter influencing the outcome of cells in culture. Here, immature and mature seeds were used to obtain first cotyledonary leaves which then served as explants. It was observed that mature seeds could give rise to cotyledonary leaves upon initiation in callus inducing media, whereas immature seeds failed to do the same. Similar reports were made by Malik et al, 2004 (*Triticum aestivum*) and by Dhavala et al, 2009 (*Solanum trilobatum).* As seeds served to be the initial source for explant, seed viability will have a bearing on the outcome of the experiments. It was observed that seeds stored at room temperature did not germinate and their cotyledons were degenerated. Unviable seeds were treated with GA10 ppm to break seed dormancy however; it did not help. Hence for all experiments seeds after collection were stored at -20°C until use. Andersone and Ievinsh, 2002 have reported the effect of cold storage on in vitro development of bud in *Pinus sylvestris* where they have shown that cold storage affects oxidative metabolism, thereby affecting the morphogenesis during *in vitro* studies.

3.3.1 Effect of Season on Somatic embryogenesis

Seasonal cues like environmental conditions also have an effect on the performance of explants in culture conditions. In this study, embryogenic calli formation was found to be higher in the months of January to April (figure 3.3 and 3.4). This is the season when fruit maturation initiates as October and November are the flowering months. In monsoon the explants in culture are less

responsive and more contamination is encountered, which is in agreement with reports of Bisht et al, 2010 for bamboo explants. Hohtola (1988) has reported higher viability and growth of explants in June and July as it represents the normal active growth period in *Pinus sylvestris*. In its study it is also commented that the season dependent metabolism is retained in tissue culture conditions and consequently, it is hard to break down the dormant state. The same difficulty may explain the lack of organogenesis in *J.curcas* as it would be difficult to switch off the callusing type of growth in cultures.

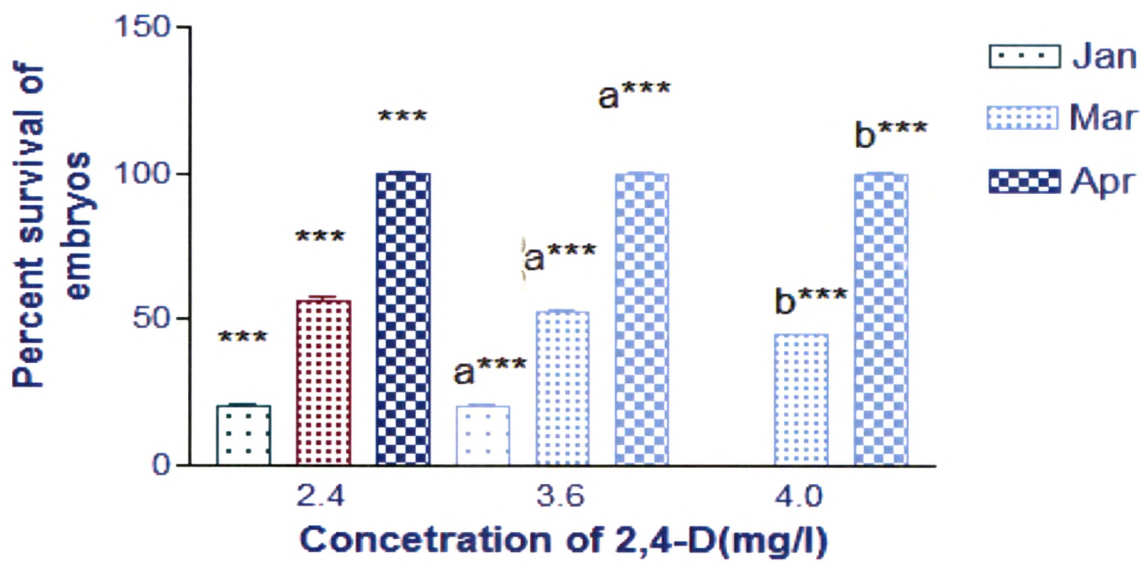

Figure 3.3: Percent Embryogenesis in MS medium supplemented with 2, 4-D

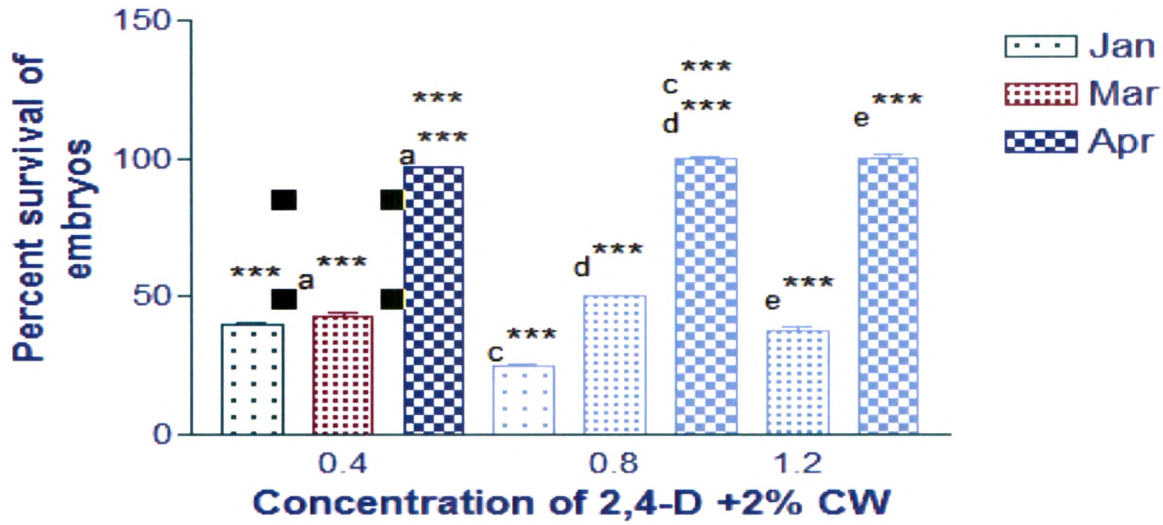

Figure 3.4: Embryogenesis in MS medium supplemented with 2, 4-D

Percent survival of embryos on MS medium supplemented with different concentration of 2,4-D/ Auxin

3.3.2 Role of Auxins

The role of auxins in morphogenesis is evident from the fact that the cells which respond to auxin revert to a dedifferentiated state and begin to divide. It was reported by Lo Schiavo et al, 1989 that auxins cause DNA to become more methylated than usual and they suggested that this might be necessary for the re-programming of differentiated cells. Tissue–specific programmes specifically associated with differentiation would become eradicated by hyper methylation, with perhaps a small fraction of the cells reaching an ultimate state of differentiation in which they become capable of morphogenesis or embryogenesis. In the present study MS medium was supplemented with two plant growth regulators (auxins) namely 2, 4-D and NAA at different concentrations. MS fortified with different levels of 2, 4-D (0.4, 0.8, 1.2, 2.4, 3.6 and 4.0mg/l) has been used for inducing embryogenic calli in *J curcas*. MS with 0.4, 0.8 and 1.2 mg/l 2, 4-D was supplemented with 2% and 10% coconut water respectively. It has been reported that the culture of explants in medium containing 2,4-D, increases the endogenous auxin levels in the responsive explants and this acts as a crucial signal determining embryogenic fate of cultured cells (Jimenez, 2005). Higher endogenous IAA concentrations have been shown in different species/explants as being associated with an increased embryogenic response. (Pasternak et al,

2002). Auxins could hence play a determining role in somatic embryo development and maturation in *Jatropha curcas*. Here we report studies on somatic embryogenesis by solid and liquid culture conditions and the role of auxin.

3.3.3 Liquid suspension culture studies

In this study, the cotyledonary leaves were initiated on MS supplemented with varied concentration of 2, 4-D (0.4, 0.8, 1.2 mg/l) and NAA (5mg/l, 10 mg/l, 15 mg/l) for a period of 30 days followed by their transfer to no auxin media. It has been reported that the presence of auxin is critical for embryo initiation and lowering of auxin concentration or its complete absence fosters maturation (Halperin and Wetherell, 1964). Hence in order to obtain somatic embryos one month old calli from explanted cotyledon were inoculated into suspension culture having only MS salts with no plant growth hormone. Cells in suspension were sub cultured at a regular interval of one week and observed microscopically at an interval of three days under 40 x. Cells were kept in suspension for a period of 30 days. Total cell count was performed and embryogenic structures were looked for by double staining (Acetocarmine+Evan's blue) using hemocytometer.

Greenish white callus started appearing at the edges of cotyledonary leaves and also at the midrib and on small veinlets. It turned to be dense mass of greenish white friable calli. After 30 days in culture, these calli were transferred to embryo inducing medium which was MS basal (liquid). It has been reported that 1.0 gram of tissue is required for initiation of suspension culture (Salaj et al, 2007).

In the current study it was observed that 350-700 mg of tissue shows good growth response in suspension culture. Once in suspension culture, it was observed that the cell number was initially low for first 4-5 days. It could be attributed to acclimation of cells in new medium. As shown in Table-3.1, the cell number increased within 10-12 days and then a gradual decrease was observed after 20 days. Similar reports have been cited by Soomro et al, 2007.

Initially cell suspension culture comprised of single cells. These cells were round and densely cytoplasmic with distinct nuclei. Some large and highly vacuolated cells with sparse cytoplasm were also observed. Early embryogenic structures like globular, heart shaped and torpedo shaped

cells were seen albeit a little late. This could be because of lingering effect of 2, 4-D on the cells as they were in prolonged 2, 4-D exposure. At four days in suspension, generally elongated cells were seen. Globular shaped cells were observed at 17th day in suspension culture. Heart shaped and torpedo shaped cells could be observed between 18-25 days time. The presence of these structures' confirmed that embryogenesis had been induced in the cells in liquid suspension (figure 3.5).

Table-3.1 Cell count after transfer of calli (30 days old) from MS+2, 4-D to MSB (liquid)

Media with 2,4-D (mg/l)	After 5 days Cells/ml	After 10 days Cells/ml	After 20 days Cells/ml	After 30 days Cells/ml	After 34 days Cells/ml
0.8	200	1213	750	212.5	150
1.2	1250	2475	2060	1625	500

Similar observations were reported with somatic embryogenesis from cotyledons of Soybean (Santos et al, 2006). The structure of somatic embryos has not been affected by culture in liquid medium. Somatic embryos in liquid medium showed similar morphology as in tissues cultured on solidified media. However, when plated on solid media, these cell aggregates failed to grow. Similar studies have been reported in Pine, wherein proliferation of embryogenic tissues occurs in liquid medium; however, the maturation is less frequent (Salaj et al, 2007). While no such cells were seen in media supplemented with various concentration of NAA. Hence, NAA as a plant growth regulator to induce somatic embryogenesis in *Jatropha curcas*, could be a less preferred substitute as compared to 2, 4-D.

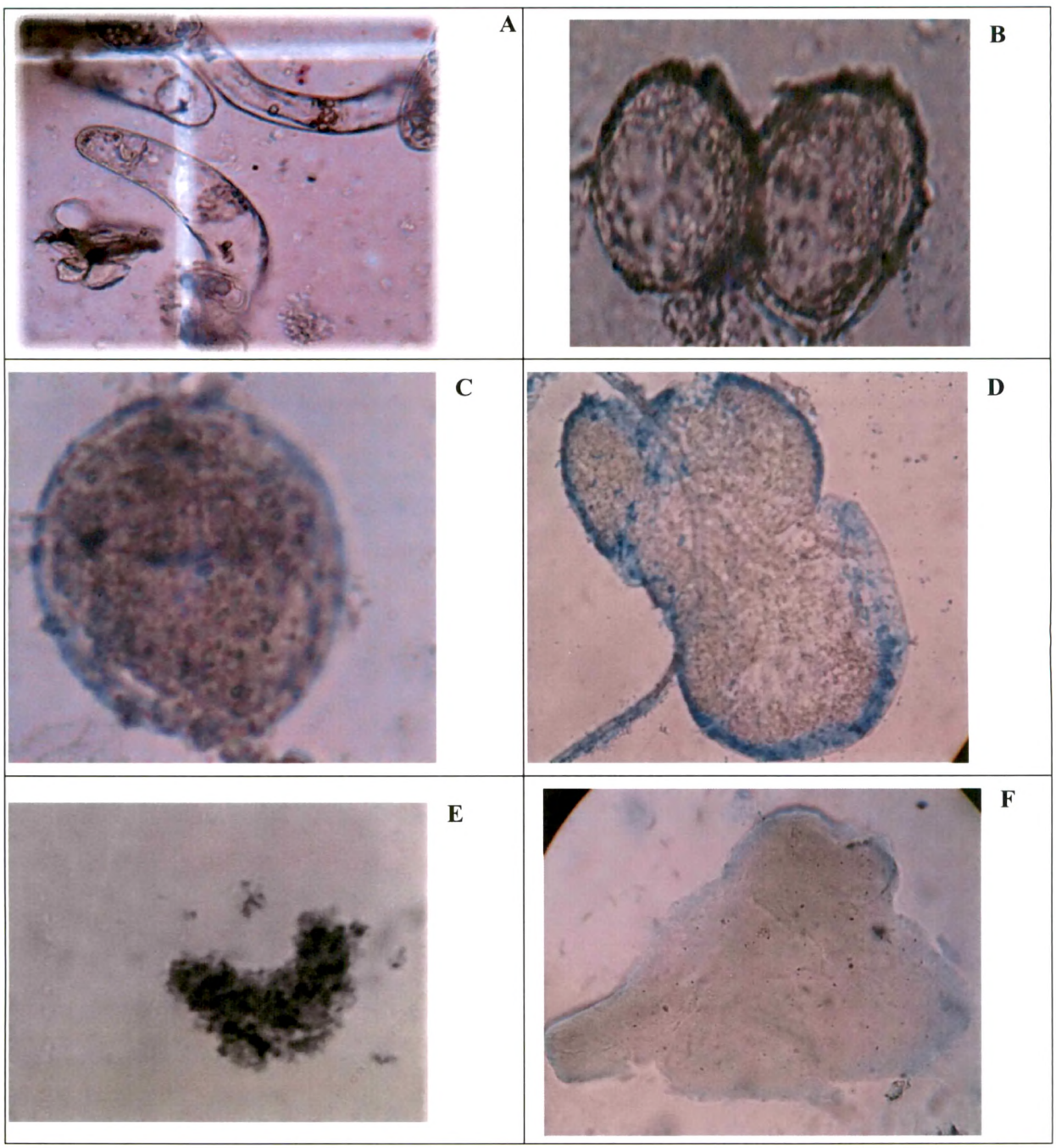

Figure 3.5: A-F: Cells seen in suspension culture;A- Elongated cells B-Round cells,C-Globular embryos,D- Cell aggregate with a globular embryo,E- Heart shaped cells ,F-Torpedo shaped cells

3.3.4 Solid media studies

Second approach was short time auxin pulse treatment wherein the cotyledonary leaves were initiated on MS +2, 4-D (0.4, 0.8, 1.2 mg/l + 2% coconut water and 0.4, 0.8 and 1.2 mg/l +10% coconut water and 2.4, 3.6 and 4.0 mg/l) and MS+ NAA for a period of seven days or fourteen days followed by their transfer to no auxin medium. In the current study different stages of somatic embryos were seen (figure 3.6).

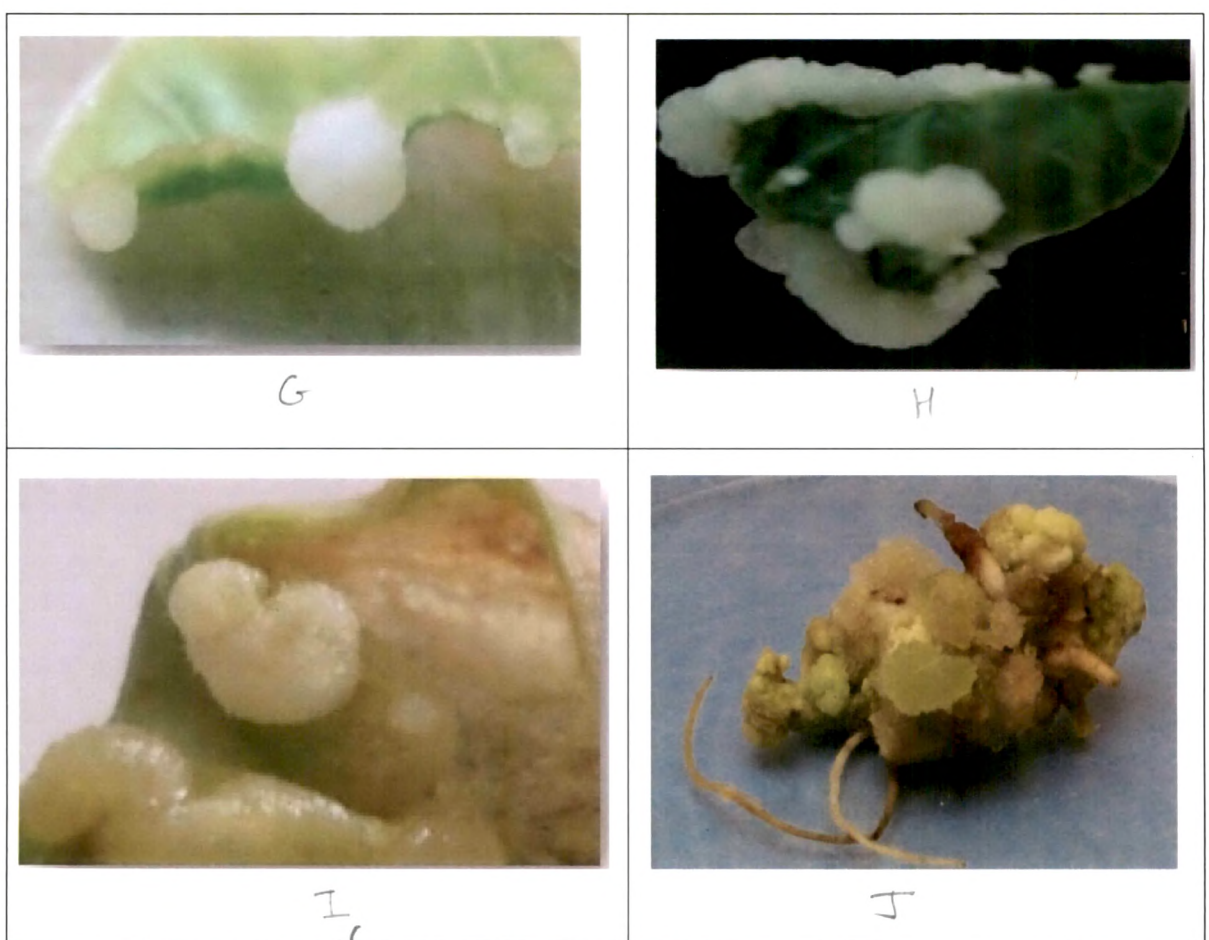

Figure 3.6: G-J: Stages in direct somatic embryogenesis as observed on solid media: G- Globular embryos seen at the edge, H: Small nodular embryos seen at the edge and veinlets, I- Heart shaped embryo, J- Direct somatic embryogenesis

Nomura and Komamine (1986) have reported that there are atleast two stages in somatic embryogenesis, stages requiring and inhibited by auxins. Hence, auxin levels were checked on day 4, day 7 and day 14. On day 4 pre-embryogenic masses were not observed whereas on day 7 pool of embryogenic and non-embyrogenic structures were observed. On day 14 most of the calli had turned embryogenic. Hence, day 4 calli was considered as non-embryogenic calli (NE) and day 7 and day 14 had both embryogenic (E) and non-embryogenic characteristics. In this set of experiment the cotyledons were cultured in MS+2, 4-D and MS + NAA for a period of 7 days and fourteen days respectively. Total auxin levels were estimated in E and NE calli (figure 3.7, 3.8, 3.9).

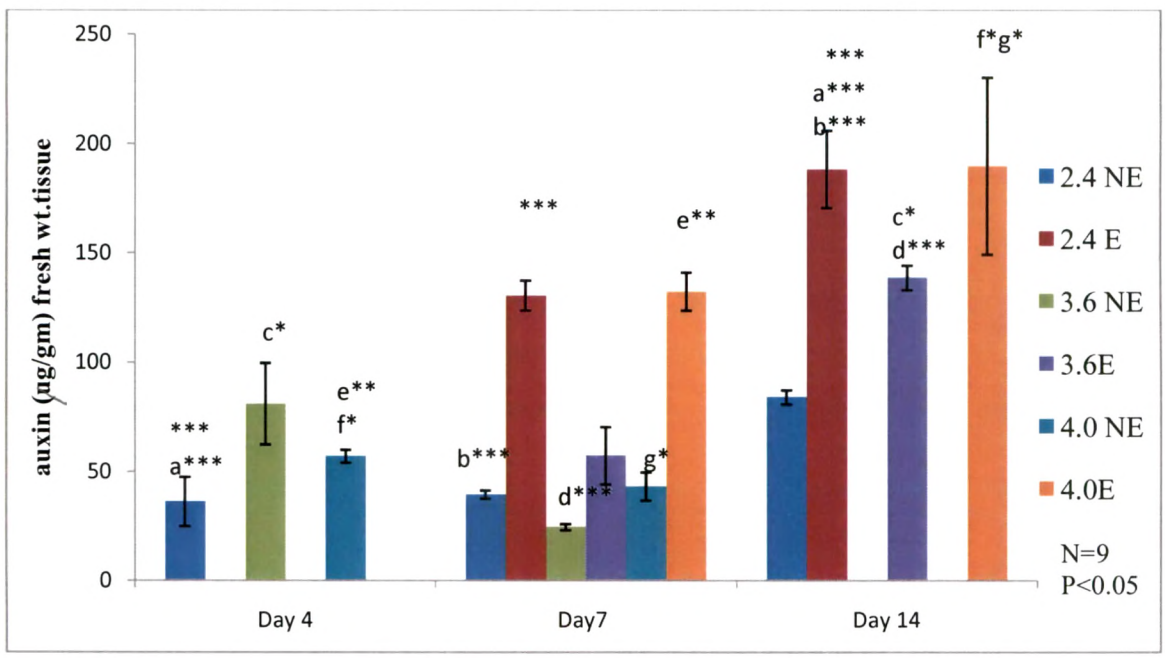

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Figure 3.7: Comparative auxin levels in MS +2.4, 3.6 and 4.0mg/l 2, 4-D

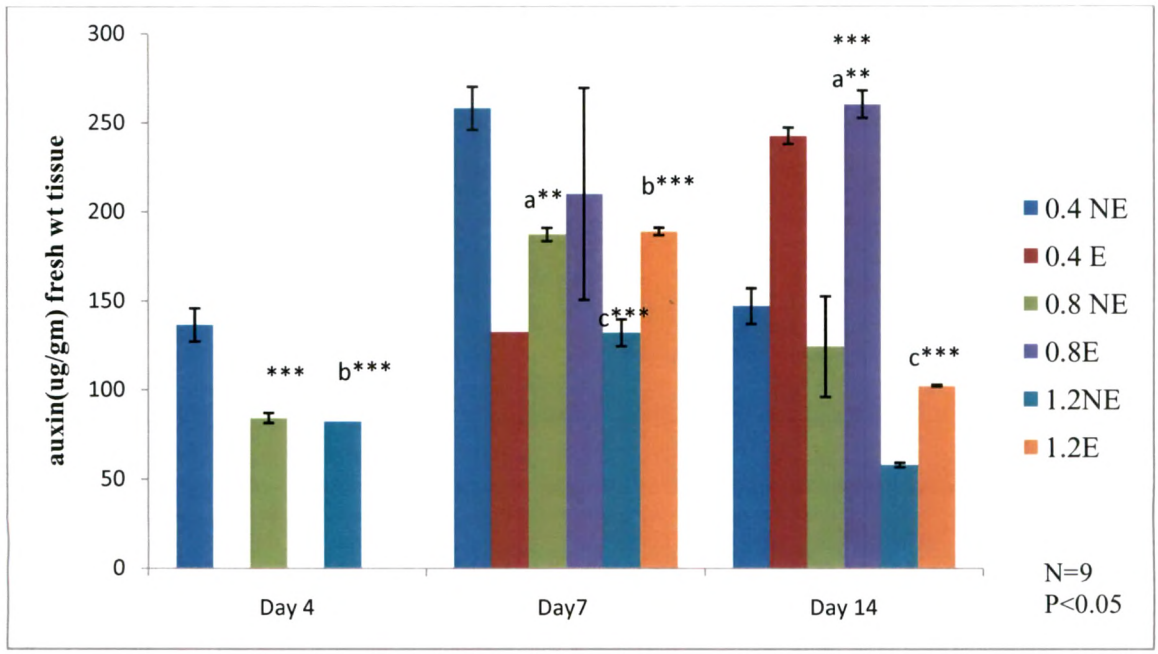

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Figure 3.8: Comparative auxin levels in MS +0.4, 0.8 and 1.2mg/l 2, 4-D+(10%)CW

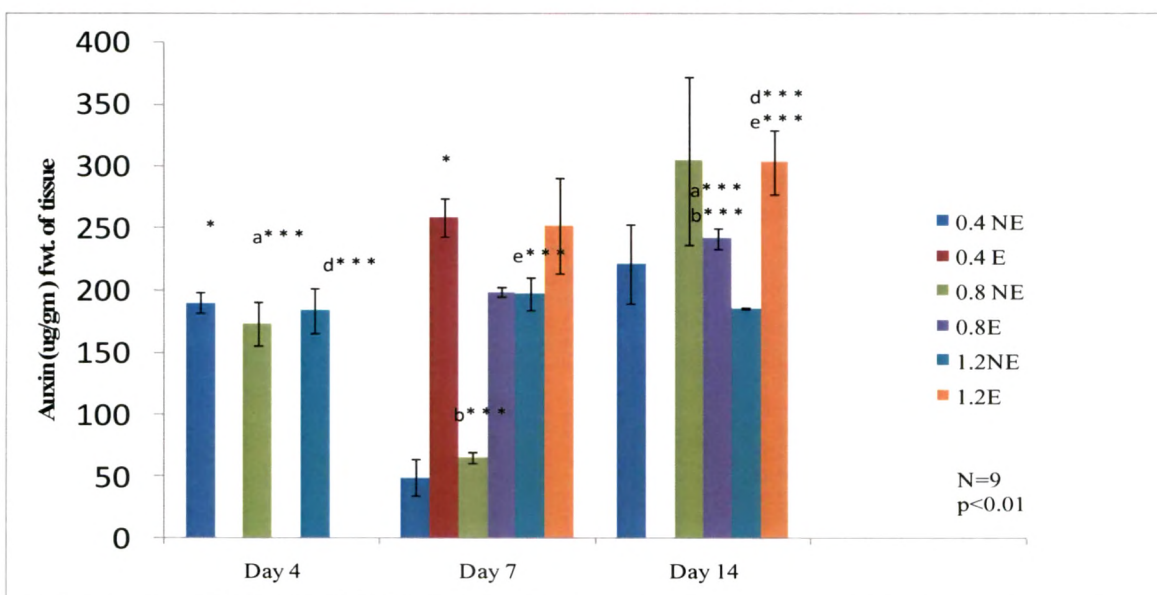

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Figure 3.9: Comparative auxin levels in MS +0.4, 0.8 and 1.2mg/l 2, 4-D+ (2%) CW

It was observed that MS fortified with higher levels of 2, 4-D devoid of coconut water could trigger higher embryo formation. Lower levels of 2, 4-D in MS medium could trigger embryogenesis only if supplemented with coconut water. Total auxin levels were estimated and it was found that a significant increase is seen in total auxin in embryogenic(E) calli(7 days and 14 days) as compared to non-embryogenic calli (NE)(4 days) in various media combinations studied. MS supplemented with 1.2 mg/l 2, 4-D + 10% CW is the only combination observed so far which shows a decrease in total auxin on day 14 in E calli (figure 3.7). Each media was observed for the number of embryos formed. It could be concluded from the present study that 2, 4-D alone at higher concentration (4.0 mg/l) is a preferred medium to induce embryogenesis in *J. curcas* as compared to lower 2, 4-D levels with coconut water. This could be due to the fact that coconut water is an undefined medium and hence may interfere with reproducibility of procedure.

This is the first report of liquid suspension culture studies of *Jatropha curcas* and direct as well as indirect somatic embryogenesis.

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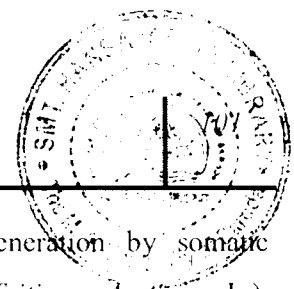

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