CHAPTER-V

DISCUSSION

Genetic engineering of plants, an integral component of plant biotechnology is one of the dynamic frontiers of biological sciences with rapidly emerging practical applications. It has the potential to provide solutions to a range of problems like pest control, biotic and abiotic stresses and increase in productivity.

Development of transgenic *J. curcas* is still a difficult problem mainly due to secretion latex from cut tissue, which makes leaf explants recalcitrant to *Agrobacterium* infection and the lack of proper regeneration protocol which is a prerequisite for genetic transformation. The major focus of the present thesis therefore, has been (a) Screening of selected *J.curcas* genotypes for regeneration potential (b) Development of reproducible regeneration protocol from leaf and petiole explant (c) Attempts to genetically transform via *Agrobacterium tumefaciens*-mediated transformation (d) Histochemical GUS assay and Molecular characterization of the transgenic micro-shoots.

Therefore, this study was initiated with an objective of developing a reproducible and efficient *in vitro* plant regeneration method from different types of explant (Leaf, petiole, cotyledonary leaf and petiole) and source of explant (*in vitro* and *in vivo*) of three toxic genotypes and a non-toxic cultivar of *J. curcas*. The study also aimed to standardize *Agrobacterium*-mediated transformation and their molecular characterization.

PART A:

5.1. REGENERATION

5.1.1. Shoot bud induction

Optimum concentrations of PGRs for shoot bud induction of toxic and non-toxic cultivar was determined using leaf and petiole explant. In the present study shoot bud induction directly from *in vitro* and *in vivo* leaf and petiole explant of toxic and non-toxic *J.curcas* could be achieved on MS basal medium supplemented with TDZ or BAP individually and in combination with IBA. The main factors that affected regeneration in our study were the concentrations and combinations of PGRs, source and type of explant, orientation of petiole explant and genotypes/cultivars. Our results were in agreement with results of others (Lin et al., 1997; Magioli et al., 1998; Geetha et al., 1998; Tyagi et al., 2001; Sharma and Wakhlu, 2001; Arockiasamy et al., 2002; Guda et al., 2003; Gubis et al., 2004; Azad et al., 2005; Sujatha et al., 2005; Feyissa et al., 2005; Ozaslan et al., 2005; Landi and mezzitti, 2006**;** Raghu et al., 2006; Lombardi et al., 2007; Reddy et al., 2008; Deore and Johnson, 2008).

The PGRs play an important role in plant morphogenesis. Huetteman and Preece (1993) have reported that TDZ is a potent cytokinin for woody plant tissue culture. The percentage response of explant forming shoot buds increased with increase in the concentration of TDZ in both toxic and a non-toxic *J.curcas*. Similar observations were made in *Alstromeria* species (Lin et al., 1997), *Solanum melongena* (Magioli et al., 1998), *Hagenia abyssinica* (Feyissa et al., 2005), *Embellia ribes* (Raghu et al., 2006) and *Jatropha curcas* (Reddy et al., 2008). Such response may be due to the increase in the level of endogenous cytokinins by the effect of the growth regulator used in the medium. Hare and Staden (1994) reported that TDZ has capacity to inhibit the action of cytokinin oxidase, which in turn may increase the level of endogenous cytokinins. In the present investigation it was observed that compact shoot buds were induced at high concentration of TDZ (1-2 mg/L) due to which shoot elongation was inhibited in subsequent culture. Lower levels of TDZ induced relatively fewer shoot buds, but these developed rapidly into shoots in subsequent culture. In contrast, media containing high concentration of TDZ had more visible primordia but, only a few developed into shoots. Nielsen et al. (1993) also observed the similar results in *Miscanthus sinensis*. In chile pepper, 10 µM of TDZ induced multiple shoots but these were small and did not elongate (Hyde and Phillips, 1996). In the present study it was observed that TDZ was more effective than BAP as cytokinin and the response of explant forming shoot bud decreased following addition of IBA to TDZ or BAP containing medium as a result of callus formation. Similar observations were reported in *Phellodendron amurense* (Azad et al., 2005), *Jatropha curcas* (Deore and Johnson, 2008; Reddy et al., 2008). Variation in the activity of different cytokinins can be explained by their differential uptake rate reported in different genomes (Blakesey, 1991), varied translocation rates to meristematic regions and metabolic processes, in which the cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds (Kaminek 1992). In the present study it was find out that 0.5 mg/L is optimum concentration of TDZ for regeneration in *J.curcas.*

Regeneration efficiency was also affected by source of explant. *In vitro* explant has more regeneration efficiency and more number of shoot buds as compared to *in vivo* explant (Guda et al., 2003; Ozaslan et al., 2005; Reddy et al., 2008). In the present study *in vitro* leaf and cotyledonary leaf and petiole explant had higher rate of regeneration efficiency and more number of shoot buds as compared to *in vivo* explant in both toxic and a non-toxic cultivar. This may be due to level of endogenous PGRs or different behavior can be related to different mechanisms of control of the endogenous PGRs metabolism. Similar results were observed in *Echinacea purpurea* (Guda et al., 2003), *Tomentosa steud* (Ozaslan et al., 2005) and *Jatropha curcas* (Reddy et al., 2008).

The frequency of adventitious shoot regeneration or shoot morphogenesis differed depending on the type of explant. Our results were in agreement with results of others (Geetha et al., 1998; Tyagi et al., 2001; Gubis et al., 2004; Lombardi et al., 2007). The regeneration efficiency and number of shoot buds were higher in cotyledonary leaf and petiole as compared to leaf and petiole from established shoot culture in both toxic and a non-toxic cultivar of *J.curcas*. This may be due to level of endogenous PGRs or different behavior can be related to different mechanisms of control of the endogenous PGRs metabolism. Similar results were observed in *Cajanus cajan* (Geetha et al., 1998), lentil (Khawar et al. 2004). The regeneration efficiency and number of shoot buds were higher in leaf than petiole explant in both toxic and a non-toxic cultivar of *J.curcas*. This may be due to level of endogenous hormones and more surface area available for leaf as compared to petiole. Similar results were observed in sugar beet (Ritchie et al., 1989), African violet (Sunpui and Kanchanapoom, 2002) and *Lycopersicon esculentum* (Gubis et al., 2004).

The orientations of petiole explant in the medium also affected the regeneration efficiency (Sharma and Wakelin, 2001; Arockiasamy et al., 2002). The regeneration efficiency and number of shoot buds were higher in horizontally placed explant as compared to vertically placed explant of both *in vitro* and *in vivo* petiole explant in both toxic and a non-toxic cultivar of *J.curcas*. Similar findings were reported in *Heracleum candicans* (Sharma and Wakhlu, 2001), *Eryngium foetidum* (Arockiasamy et al., 2002). This may be due to little contact of explant to medium in vertical position as compared to horizontal position.

Genotype/cultivar is one of the most important factors affecting regeneration (Feyissa et al. 2005; Landi and mezzitti, 2006). The regeneration efficiency and number of shoot buds were higher in toxic cultivar as compared to non-toxic cultivar in both leaf and petiole explant. In our study, it was also observed that, all the three genotypes of toxic and a non-toxic cultivar showed differences in percentage of shoot bud induction, number of shoot buds per explant and elongation of regenerated shoot buds. This different behavior can be related to different mechanisms of control of the endogenous PGRs metabolism and/or contents. Similar results were reported in Mulberry species (Chitra and Padmaja, 2005), *Hagenia abyssinica* (Feyissa et al., 2005) and Fragaria (Landi and Mezzitti, 2006). Genotypic/cultivar effect on shoot regeneration and elongation have been described in many species, and could be due, in part, to differences in the levels of endogenous hormones, particularly cytokinins levels during the induction period although the precise mechanism remains unclear (Pellegrineschi, 1997; Schween and Schwenkel, 2003). Henry et al. (1994) reported that genotypic differences with respect to embryogenesis and regeneration result from quantitative or qualitative genetic differences.

5.1.2. Shoot proliferation and elongation of regenerated shoot buds

The inhibitory effect of high concentration of TDZ on shoot elongation has been reported by many researchers and recommend that the TDZ concentration must be reduced and/or other cytokinins or combinations of cytokinins and auxins must be used for further shoot elongation (Preece and Imel, 1991; Feyissa et al., 2005; Raghu et al., 2006), and our results are in agreement with above findings. Therefore, the clump of induced shoot buds from leaf and petiole explant was transferred to a medium containing 2 mg/L Kn, 1mg/L BAP and 1 mg/L NAA for shoot proliferation. Percentage of shoot proliferation increased at low concentration of TDZ as compared to high concentration of TDZ and also more percentage of shoot proliferation has been observed on BAP contaning medium as compared to TDZ contaning medium (Reddy et al., 2008). This difference in shoot proliferation in the present study may be due to level of endogenous PGRs, or different behavior can be related to different mechanisms of control of the endogenous PGRs metabolism. It was also observed that shoot proliferation was higher in toxic as compared to non-toxic. As compared to petiole, shoot proliferation was more in leaf explant and when compared with cotyledonary leaf, shoot proliferation was low in leaf of shoot culture in both toxic and a non-toxic cultivar's of *J.curcas.* These results were in agreement with earlier reports (Geetha et al., 1998; Tyagi et al., 2001; Gubis et al., 2004; Lombardi et al., 2007; Feyissa et al., 2005; Landi and mezzitti, 2006). Proliferated shoots were separated and transferred to elongation medium. The elongation of individual shoots depends up on the concentrations and combinations of PGRs in the medium. Elongation was more in leaf explant as compared to petiole explant and also more in cotyledonary leaf as compared to leaf. As compared to toxic shoot elongation was poor in non-toxic cultivar. This difference in shoot elongation in the present study may be due to level of endogenous PGRs, or different behavior can be related to different mechanisms of control of the endogenous PGRs metabolism (Reddy et al., 2008). The maximum elongation was obtained in BAP and IAA combinations as compared to BAP and IBA, and BAP and NAA combinations. The best elongation was obtained in medium containing 0.5 mg/L BAP and 1.5 mg/L IAA. Our results are in agreement with the previous reports (Christopher and Rajam 1996; Venkataiah et al. 2003). Shoot elongation decreased in the medium containing BAP and IBA, may be due to proliferation of more axillary buds. Similar observation has been observed in *Eupatorium triplinerve* (Martin 2003). The least elongation was observed in a medium containing BAP and NAA may be due to the profuse callusing at the basal end of proliferated shoots (Kumar et al., 2008; Koroch et al., 2002).

5.1.3. Rooting and acclimatization

Optimum concentrations and combinations of auxins for rooting of toxic and non-toxic cultivar were determined. Auxins, strength of MS medium, culture type and cultivars significantly affected the rooting percent. Our results were in agreement with results of others (Vuylasteker et al., 1998; Ahmed et al., 2006; Singh et al., 2008). It is well reported that auxins are a potent hormone for rooting (Sujatha and Mukta, 1996; Vuylasteker et al., 1998; Ahmed et al., 2006; Nandagopal and Kumari, 2007; Singh et al., 2008; Reddy et al., 2008). Maximum rooting percentage was observed on hormone free half strength solid MS medium after pulse treatment of grownup shoots with half strength MS medium supplemented with 3 mg/L IBA, 1 mg/L IAA and 1 mg/L NAA. Significant differences were observed between toxic and non-toxic cultivar however, no significant difference were observed between different genotypes of toxic *J.curcas*. This difference in rooting percentage in toxic and non-toxic cultivar in the present study may be due to level of endogenous PGRs, or different behavior can be related to different mechanisms of control of the endogenous PGRs metabolism and/or contents. More rooting percentage were observed on hormone free solid basal MS medium after pulse treatment with auxins as compare to directly cultured on auxins containing solid MS medium (Singh et al., 2008; Reddy et al., 2008). Higher rooting percentage was observed on half strength basal MS medium as compared to full strength MS medium (Singh et al., 2008; Reddy et al., 2008). The acclimatization of the rooted shoots was accomplished and approximately 90% of the plants were successfully transferred to polybags in greenhouse conditions.

PART B:

5.2. GENETIC TRANSFORMATION

The effect of increasing concentrations of hygromycin was assessed separately on leaf explant and *in vitro* developed shoots. Hygromycin was highly effective even at low concentration (2.5 µg/ml). About 70-78% of the explant/shoot was bleached by 30th day in culture medium containing 2.5 µg/ml hygromycin. The phytotoxic effect of hygromycin was noticeable at 5 µg/ml in the medium as explant/shoot started to bleach between 10 days and 15 days following inoculation, resulting in a total loss of chlorophyll pigmentation (bleaching) by day 30 days. Similar response of explant to bactericidal effect of hygromycin has been observed in *J.curcas* (Li et al., 2008). Three antibiotics namely, sporidex, carbenicillin and cefotaxime were used at various concentrations (250, 500, 750 and 1,000 mg/L) to eliminate the background *Agrobacterium*. They also had a detectable effect on shoot organogenesis. In the present study, 500 mg/L cefotaxime in the medium had a most suitable effect on bacterial elimination without adversely affecting morphogenesis. Cefotaxime, which was used for suppressing *Agrobacterium*, was found to severely inhibit shoot regeneration from root explant of Arabidopsis (Valvekens et al., 1988). In the case of *J. curcas*, cefotaxime did not inhibit both callus induction and shoot regeneration capacity of its cotyledon explant and, therefore, cefotaxime was used to inhibit *Agrobacterium* after co-cultivation with cotyledon explant (Li et al., 2008).The response of explant to bactericidal antibiotics has been observed in many crops(*Cajanus cajan* Lawrence and Koundal 2000; Shrivastava et al., 2001, *Glycine max*, (Meurer et al., 1998; Santarem et al., 1998) *Vigna unguiculata* (Muthukumar et al., 1996), *Camelia sinensis* (Mondal et al., 2001), *Lathyrus sativus* (Barik et al., 2005) and *Jatropha curcas* (Li et al., 2008).

The enzymatic activity of GUS was substantiated by the positive histochemical assay, as evidenced by the blue coloration, which developed on leaf explant. The explant which survived in the selection medium containing 5 µg/ml hygromycin was GUS-expressive.This construct has already been safely and successfully exploited in the *Agrobacterium*-mediated genetic transformation of several woody tree species, including almond (*Prunus dulcis*; Miguel et al., 1999), “Royal Gala” apple (Malus sp.; Liu et al., 1998), citrange (*Citrus sinensis*; Cervera et al., 1998), lime orange (*Citrus aurantifolia*; Pena et al., 1995) and rubber (*Hevea brasiliensis*; Arokiaraj et al., 1998).

Leaf explant that was directly infected with *Agrobacterium* without pre-culture in regeneration medium showed a lower transformation competence than the ones cultured. Leaf explant pre-cultured for 4 days had the highest transformation competence (11.32%). Shorter durations of preculture (1-3 days) elicited a lower shoot organogenic response, while longer durations (5-7days) resulted in more nontransformed shoots. Explant pre-culture has been reported to be a useful procedure in *Agrobacterium*-mediated transformation of several plant species, such as *Cicer arietinum* (Kar et al., 1996), *Cajanus cajan* (Lawrence and Koundal, 2000), *Vigna mungo* (Karthikeyan et al., 1996), *Camelia sinensis* (Mondal et al., 2001) and *Lathyrus sativus* (Barik et al., 2005).

At the late-log phase, corresponding to OD_{600}= 0.6, we obtained the maximum transformation (13.55 %) as measured by the surviving shoots in selective medium expressing the marker genes and tested positive by PCR. An increase or decrease in the optical density of the *Agrobacterium* inoculum was not conducive to transformation, while extensive tissue damage occurred at optical density values greater than 1.0 because of bacterial overgrowth. A late log phase corresponding to an OD of 0.6 was the most effective for obtaining high rates of transformation in almond (Archilletti et al., 1995), *Camelia sinensis* (Mondal et al., 2001), *Camelia sinensis* (Kumar et al., 2004) and *Lathyrus sativus* (Barik et al., 2005).

There is secretion of various phenolics compounds from the wound sites of leaf explant, which inhibits the transformation efficiency. The maximum transformation efficiency (8.99%) was scored using intact (unwounded) leaf explant. When leaf explant was injured with either glass beads or hands pricking with hypodermic needle transformation efficiency decreased with a high rate of tissue browning. Thus, wounding was not only unnecessary for inducing transformation but also deleterious to regeneration. This was also found to be the case for Datura (Ducrocq et al., 1994), walnut (McGranahan et al., 1988), *Camelia sinensis* (Mondal et al., 2001, Kumar et al., 2004). Potrykus (1990) and Barik et al. (2005) reported that wounding and wound-induced cell division was required for the transformation of Dicot plants and *Lathyrus sativa* respectively.

A range of bacterial cell densities (10^7-10^{10} cells/ml) adjusted by diluting the *Agrobacterium* suspension, was evaluated for explant infection. Optimum results were obtained with a density of 10^9 cells/ml. Of the range of increasing durations tested for explant infection with a diluted *Agrobacterium* culture, the maximum transformation efficiency (12.67%) was recorded with a 20 min long treatment at a bacterial cell density of 10^9 cells/ml. The requirement for an ideal bacterial density has been shown to vary with plant species. Effective bacterial densities have been reported to be 4×10^7 cells/ml for citrus (Pena et al., 1995), 10^7 cells/ml for hybrid poplar (Howe et al., 1994), 10^9 cells/ml for *Galega orientalis* (Collen and Jarl, 1999), 10^8 cells/ ml for *Cajanus cajan* (Singh et al., 2002), 1.5×10^8 cells/ml for Cicer arietinum (Husnain et al., 1997) and 5×10^8 cells/ml for *Vigna unguiculata* (Muthukumar et al., 1996).

The co-cultivation period and medium pH play a key role in genetic transformation of plants. With a view to determining the suitable period for co-cultivation, leaf explant was co-cultivated with above mentioned *Agrobacterium* strains for an increasing length of time (1-7 days). Co-cultivation for 2-7 days is generally considered to be suitable for *Agrobacterium*-mediated transformation, as reported for many plant species. More than 5 days of co-cultivation encouraged an overgrowth of bacteria with a concomitant decrease in transformation efficiency in garden pea (De Kathen and Jacobsen, 1990) and flax (Dong and McHughen, 1993). The maximum transformation efficiency (12.84%) was achieved after 4 days of co-cultivation. A co-cultivation period of 4 days was found to be suitable for *Galega orientalis* (Collen and Jarl, 1999), whereas a 2-day co-cultivation period was determined to be the best for several other crops, like *Cajanus cajan* (Shrivastava et al., 2001), *Cicer arietinum* (Kar et al., 1996; Husnain et al. 1997) and *Vigna mungo* (Karthikeyan et al. 1996). Co-cultivation periods longer than 4 days were unsuitable because of uncontrollable over-growth of bacteria. Co-cultivation for more than 3 days led to a decrease in transformation efficiency and about 50% explant was lost because of bacterial overgrowth in experiments designed to produce transgenic Datura (Ducrocq et al. 1994). Explant co-cultivated for a shorter period (1-3 days) produced shoots, but only a few of these were resistant to hygromycin (3.43-7.65%). The differential requirement of co-cultivation period largely depends upon the *Agrobacterium* strain used or the medium for bacterial culture or co-cultivation.

A co-cultivation medium pH of 5.6 was found to be the best with respect to transformation frequency, with a decrease in transformation frequency occurring either below or above this pH threshold value (5.6) of the co-cultivation medium. The effect of the pH of the co-cultivation medium on transformation efficiency in grain legumes is well documented. Aliev et al. (1988) observed that the virulence of *A. tumefaciens* was related to the pH of the leaf and stem cell sap in cotton (*Gossypium hirsutum* cv. 109F), with the best infectivity being achieved at pH 5.5. Shrivastava et al. (2001) and Husnain et al. (1997) found a pH of 5.6 to be the most effective for transforming *Cajanus cajan* and *Cicer arietinum*, respectively, whereas Meurer et al. (1998) found a pH of 5.5 to be the best for transforming *Glycine max*.

The acetosyringone concentration plays a key role in genetic transformation of plants (Bolton et al., 1986; Shimoda et al., 1990; Somleva et al., 2002; Vasudevan et al., 2007; Lopez et al., 2004). To determine the right concentrations of acetosyringone for high-efficiency transformation, we used different concentrations of acetosyringone (50, 100, 200 and 400 μM). Maximum rate of transformation efficiency (9.92 %) was obtained at 100 μM as measured by the surviving shoots in selective medium expressing the marker genes and tested positive by PCR. Acetosyringone (100 μM) was found to be the best with respect to transformation efficiency. These results were in agreement with earlier reports (Bolton et al. 1986; Shimoda et al. 1990; Somleva et al. 2002; Vasudevan et al. 2007; Lopez et al. 2004)

DNA obtained from several independent hygromycin resistant, GUS-positive lines revealed predicted amplification products of 400 bp and 866 bp with GUS and DREB2A gene-specific primers, respectively. This indicated that the presence of both the linked marker transgenes GUS and DREB2A as a single T-DNA in the transformed genome. No amplification product was detected in DNA from untransformed shoots when subjected to PCR amplification with either of the two primers. A differential response to gene integration was evidenced from dot blot analysis which is due to differences in their transcriptionally active regions or hot spots as T-DNA from *Agrobacterium* is known to be generally inserted into the transcriptionally active regions of host genome (Koncz et al., 1989; Ingelbrecht et al., 1991). Different hybridization pattern among three analysed transformed plants indicated that the T-DNAs randomly integrated into *J.curcas* genome (Kotsuka and Tada, 2008).

After transformation, regenerated putative transformed buds proliferated on medium containing 2 mg/L Kn, 1 mg/L BAP and 1 mg/L NAA. Individual (0.3-0.5 cm) shoots were separated from the clump of proliferated shoots and transferred to elongation medium where approx 2.5 cm elongation was achieved after 6 weeks. About 40% rooting was observed on half strength MS medium supplemented with 2% sucrose and 3 mg/L IBA, 1 mg/L IAA, 1 mg/L NAA and 0.25 mg/L activated charcoal for rooting. After 6-8 weeks, 50-60% of plants survived.