CHAPTER 4: DISCUSSION

Plant tolerance to salt stress is a multigenic trait and requires the coordinated action of several genes. But it is evident by several reports that overexpression of a single gene can also impart salt tolerance to plants. In the present study, we have successfully transformed *J. curcas* with salt-responsive *SbNHX1* gene for its enhanced salt tolerance. This objective was achieved via two major transformation methods *i.e.* *Agrobacterium* mediated and microprojectile bombardment mediated methods. Before proceeding to genetic transformation with any important gene and further improvement strategies, optimization of an efficient regeneration and genetic transformation protocol is a crucial criterion to produce required sum of improved plants in limited area.

4.1 Regeneration of *Jatropha curcas*

Efficient *J. curcas* regeneration was optimized using different PGRs for leaf, cotyledonary leaf and embryo axis explants that were further used for genetic transformation. Leaf has been a preferred explant for regeneration because of its suitability for adventitious shoot regeneration and *Agrobacterium* mediated transformation (Landi and Mezzetti, 2006; Deore and Johnson, 2008; Kumar *et al.*, 2010). Juvenile plant tissues such as embryo axis, hypocotyl, epicotyl and cotyledon are highly responsive compared with mature and differentiated tissues and therefore, are commonly used plant parts for regeneration and genetic transformation via different methods (Sailaja *et al.*, 2008; Purkayastha *et al.*, 2010; Varshney and Johnson, 2010; Singh and Tiwari, 2012). BAP and IBA with a higher cytokinin/auxin ratio in the medium induced callus, and regeneration was achieved via significant callusing due to rapid cell proliferation and morphogenesis. BAP and IBA in combination for callus
mediated regeneration have been used by many researchers using different explants i.e. hypocotyl, petiole, leaf (Sujatha and Mukta, 1996), cotyledonary disc (Li et al., 2008b) and immature embryos (Varshney and Johnson, 2010).

By including TDZ in the shoot induction medium (SIM), callus formation was reduced significantly and adventitious shoot buds were formed. This result is in compliance with earlier reports for *Jatropha* using similar PGRs combination for leaf explants (Deore and Johnson, 2008; Khurana-Kaul et al., 2010). Highest shoot induction response was with 2.22 μM BAP, 2.27 μM TDZ and 0.49 μM IBA for leaf (Table 3.1) as well as embryo axis (Table 3.3). For *Jatropha*, TDZ alone or in combination with IBA and BAP + IBA has been shown to induce direct shoot buds without formation of intervening callus from various explants in previous reports (Sujatha et al., 2005; Kumar and Reddy, 2010; 2012; Sharma et al., 2011). TDZ is reported to be effective in adventitious shoot induction in many other plant species also (Landi and Mezzetti, 2006; Lata et al., 2009; Ma et al., 2011). Sujatha et al. (2005) found TDZ optimum for shoot multiplication from axillaries while efficient adventitious shoot regeneration from leaf tissues was achieved on medium with BAP and IBA. Some reports are contradictory to other recent reports that reveal TDZ is meant for direct shoot bud induction and not for callus induction in *Jatropha*. Purkayastha et al. (2010) reported that shoot apices cultured on TDZ supplemented media showed a drastic reduction in shoot emergence and Li et al. (2008b) stated that TDZ is efficient PGR for callus induction but not for shoot regeneration from leaf. TDZ was not used in further subcultures after shoot induction because of its inhibitory effects in shoot formation and rooting at later stages of growth (Feyissa et al., 2005, Kumar and Reddy, 2010).

Induced shoot buds were multiplied and elongated into shoots following transfer to the MS medium supplemented with 2.22-4.44 μM BAP, 0.49-1.47 μM IBA and 1.45
μM GA₃. Shoot regeneration response was 85% and 90%, and number of shoots per explant was 6.4 and 6.7 for leaf and embryo axis respectively due to inclusion of GA₃. Addition of GA₃ in shoot regeneration medium (SRM) increased shoot proliferation, number of shoots and favoured shoot elongation (Deore and Johnson, 2008; Li et al., 2008b; Purkayastha et al., 2010; Kumar and Reddy, 2010; 2012; Mao et al., 2011; Sharma et al., 2011). Similar response was also observed in other plant species (Lata et al., 2009; Singh and Tiwari, 2012).

Root initiation, hardening and acclimatization are important for establishment of in vitro regenerated plants. For *J. curcas*, mostly ½ strength MS medium with 0.98-2.46 μM IBA has been used and rooting response was 30-80 % (Deore and Johnson, 2008; Li et al., 2008b; Varshney and Johnson, 2010; Khurana-Kaul et al., 2010; Purkayastha et al., 2010; Khemkladngoen et al., 2011). In the present study, 1.47 μM IBA is found optimum for rooting within 28-40 days. A pulse treatment of 14.7 μM IBA improved the rooting efficiency. Addition of activated charcoal in the medium further improved rooting and the condition of the plantlets to some extent by adsorption of phenolic compounds secreted by the cultures, although in young cultures, the quantity of phenolic compounds is less.

### 4.2 Salt sensitivity of *J. curcas*

Salt sensitivity of *J. curcas* was checked *in vitro* and in hydroponics. In both experiments, gradual increment of NaCl concentration after 50 mM adversely affected the germination and growth of the seedlings. At 100 mM and above, chlorophyll content of seedlings was visibly reduced upto negligible and stunted growth was clearly noticeable. Although the detailed analytical and statistical studies were not carried out but there are many recent reports with analytical measures that show similar results
Silva et al. (2011) studied the salt sensitivity of young plants at 100 mM NaCl concentration and observed that plants are sensitive to high salinity, showing high leaf Na\(^+\) and Cl\(^-\) concentrations and very low K\(^+\)/Na\(^+\) ratios after 14 days exposure of NaCl. Díaz-López et al. (2012) observed that *Jatropha* plants irrigated with NaCl levels of over 30 mM shows a significant reduction in growth by 5.82% for every 10 mM of increment in NaCl concentration. Fujimaki and Kikuchi (2010) stated that *Jatropha* is not more salt tolerant than major crops such as soybean or wheat. Hence, it is clear from these reports that salinity tolerance of *Jatropha* needs to be addressed and enhanced further.

### 4.3 Genetic transformation of *J. curcas* with *SbNHX1* gene

#### 4.3.1 *Agrobacterium* mediated genetic transformation

Transgenic plants of *J. curcas* were developed via *A. tumefaciens* mediated transformation using leaf, cotyledonary leaf and embryo axis explants. To overexpress *SbNHX1* gene in *Jatropha*, EHA105 strain of *A. tumefaciens* mobilized with the recombinant binary plasmid pCAMBIA1301-*SbNHX1* was used as vector for genetic transformation.

Li et al. (2006) reported that cotyledons reveal more susceptibility to *Agrobacterium* mediated transformation than other explants such as hypocotyls, epicotyls, petioles or leaves and choice of explants is very limited. Leaf and juvenile explants like embryo axis and cotyledonary leaf were chosen that could give higher transformation efficiency.

The optimized conditions for *Agrobacterium* mediated transformation were 0.6 bacterial culture O.D. at 600 nm, 100 μM acetosyringone in bacterial infection solution.
as well as co-cultivation medium (SIM), 20 min bacterial infection time, 3 days co-cultivation, un-wounded leaf discs with cut edges while embryo axes with 4-6 scars with blade. For *Jatropha*, many of these conditions have been reported previously (Li *et al.*, 2008b; Kumar *et al.*, 2010; Pan *et al.*, 2010; Mao *et al.*, 2011). Cefotaxime is an *Agrobacterium* inhibitory antibiotic, and in the case of *J. curcas*, cefotaxime did not suppress plant regeneration (Li *et al.*, 2008; Kumar *et al.*, 2010). Therefore, cefotaxime was used to inhibit *Agrobacterium* after co-cultivation in decreasing order from 500 to 300 mg/l during subcultures.

Transformation efficiencies followed by plant transformation and hygromycin selection were 34.81%, 26.41%, 20.85% & 29.24% and overall regeneration efficiencies were 1.62%, 1.02%, 0.48% & 1.25% for embryo axis, *in vitro* leaf, *in vivo* leaf and cotyledonary leaf explants respectively (Table 3.10, A8-A11). This is clearly noticeable that juvenile explants like embryo axis and cotyledonary leaf showed higher transformation efficiency and overall regeneration efficiency than mature explants like leaf. *In vivo* leaf showed lowest efficiency among all the explants in both transformation methods. This might be due to the secretion of latex, which hampers the *Agrobacterium* infection, and surface sterilization of *in vivo* explants may affect its proper regeneration ability. *Jatropha* cotyledonary leaf has been used by many researchers recently (Li *et al.*, 2008b; Pan *et al.*, 2010; Khemkladngoen *et al.*, 2011; Mao *et al.*, 2011; Qu *et al.*, 2012; Tsuchimoto *et al.*, 2012; Kajikawa *et al.*, 2012), but other explants from seeds were not tested so far. In this study, embryo axis showed higher transformation and overall regeneration efficiency than cotyledonary leaf. Therefore, embryo axis and other related juvenile explants *i.e.* hypocotyl and epicotyl could be used for more efficient genetic transformation of *J. curcas*. RT-qPCR is recently being used to determine copy number of integrated gene because of its high sensitivity and requirement of very low
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amount of genomic DNA and this technique has been used in many recent reports (Casu et al., 2012; Jha et al., 2012). In our results, transgenic line JL18 showed single copy gene insertion.

Out of the two latest and only reports on transgenic *J. curcas* for trait improvement, Qu et al. (2012) developed marker-free transgenic plants with increase in high quality seed oil by down-regulation of seed-specific *JcFAD2-1* gene. In another report, transgenic *Jatropha* plants overproducing glycine betaine were developed for enhanced drought tolerance (Tsuchimoto et al., 2012), but this study is at primary level and needs to be studied further. In this study, *Agrobacterium* mediated genetic transformation of *J. curcas* was successfully carried out with *SbNHX1* gene and transgenic plants were developed. Till date, several important plants have been transformed via *Agrobacterium* with *NHX1* gene from different source plants and its overexpression showed enhanced salt tolerance (Rajagopal et al., 2007; Chen et al., 2008; Bhaskaran and Savithramma, 2011).

4.3.2 Microprojectile bombardment mediated genetic transformation

4.3.2.1 Optimization of physical parameters

Gene introgression by particle bombardment is most efficient and consistent, genotype independent versatile physical process with no biological constraint (Altpeter et al., 2005). A simple and efficient microprojectile mediated genetic transformation method in *J. curcas* was established with 44.7% transformation efficiency. Different physical parameters need to be carefully examined in particle bombardment that could enhance transient GUS expression and lead to stable integration of the introduced genes (Sailaja et al., 2008). In this study, different physical parameters such as microcarrier size, velocity of particle delivery and microprojectile travel distance were optimized individually and in combination by considering the frequency of transient *gus*
expression and survival of putative transformants. Microprojectile bombardment with embryonic axis as target tissues has been used for the production of successful transgenic lines in many plants *i.e.* soybean (McCabe *et al.*, 1988; Rech *et al.*, 2008), cowpea (Ivo *et al.*, 2008), peanut (Brar *et al.*, 1994; Livingstone and Birch, 1999), castor (Sailaja *et al.*, 2008), corn (Lowe *et al.*, 2009) and cumin (Singh *et al.*, 2010).

Microprojectile bombardment is independent to any kind of target tissue but in this study embryo axes, pre-cultured for 5 days on MS medium were taken because both castor and *Jatropha* belong to Euphorbiaceae family and embryo axis was found suitable for regeneration (Sujatha and Mukta, 1996) and stable genetic transformation in castor (Sailaja *et al.*, 2008). Embryo was pre-cultured for 5 days to maximize the probability of stable transformation as actively dividing cells have ability to survive and grow under stress imposed during bombardment process (Sailaja *et al.*, 2008).

Frequency of transient *gus* expression was significantly increased (*p*<0.05) and frequency of shoot survival decreased (*p*<0.01) with an increase in particle size. Similarly, transient *gus* expression was increased while frequency of shoot survival decreased concomitantly with helium pressure. High pressure and large microcarriers penetrated into deep cell layers and integrated with genome, hence more transient *gus* expression was observed. Simultaneously, it also imposed injury leading to decrease in probability of shoot survival. The optimum He pressure was observed 1100 and 1350 psi and microcarrier size 1.0 µm independent to microprojectile travel distance, while comparing transient *gus* expression and frequency of shoot survival. In this combination, gene introgression was efficient for transient *gus* expression leading to maximum shoot survival.

Decline in transient *gus* expression with increase of travel distance in combination with microcarrier size independent to He pressure could be due to deceleration of the
microprojectile velocity. However, contrary result was observed in combination with He pressure independent to microcarrier size because of acceleration of microcarrier with high pressure. While comparing transient \textit{gus} expression and frequency of shoot survival, optimum travel distance was observed 9 and 12 cm for He pressure 1100 and 1350 psi respectively, independent to microcarrier size. The genetic transformation efficiency was determined at different microcarrier size, He pressure and target distance both in terms of transient \textit{gus} expression and shoot survival. The study revealed that transient \textit{gus} expression is not key to analyze transformation efficiency. In spite of a very high frequency of transient \textit{gus} expression at 1550 psi for 1.6 µm microcarrier, the frequency of surviving explants drastically declined during selection because high helium pressure increased particle acceleration and subsequent target tissue penetration leads to injury by the DNA coated microcarriers.

Overall optimum parameters observed were microcarrier size 1.0 µm, He pressure 1100 and 1350 psi with target distance 9 and 12 cm respectively by comparing transient \textit{gus} expression and frequency of shoot survival. The highest efficiency of transformation obtained through particle gun gene transfer in the present study is 44.7\% with optimized parameters, which is higher than earlier reports for \textit{Jatropha} (Li \textit{et al.}, 2008b; Kumar \textit{et al.}, 2010). Purkayastha \textit{et al.} (2010) transformed \textit{Jatropha} using shoot apices by particle bombardment but transformation efficiency was not reported. Higher efficiency of transformation through particle gun bombardment could probably be due to the higher number of explants tried with varying individual and in combination physical parameters. High transformation efficiency over previous methods makes optimized protocol (Joshi \textit{et al.}, 2011) efficient and thus has the potential to facilitate the genetic modification for trait improvement. The optimized parameters were used previously for evergreen coniferous tree Norway spruce (Walter \textit{et al.}, 1999), maize
(Bohorova et al., 1999), rice (Cho et al., 2004), potato (Ercolano et al., 2004), St. John’s wort (Franklin et al., 2007), Madagascar periwinkle (Guirimand et al., 2009), \textit{Jatropha} (Purkayastha et al., 2010) and cumin (Singh et al., 2010). Callus browning is a typical feature of callus cultures derived from the hypocotyl of \textit{J. curcas} (He et al., 2009) and during selection on hygromycin medium, non-transgenic tissues gradually turned brown, while putative transformed sectors remained green and showed slow growth. Brown tissues result in decreased regenerative ability, poor growth and subsequent death. Similar to our study, optimized stable transformation has been reported in different plants in recent years with higher transformation efficiency (Batista et al., 2008; Ivo et al., 2008; Jagga-Chugh et al., 2011; Liu and Godwin, 2012)

Histochemical GUS assay after the final selection of transformed lines, showing constitutive expression of \textit{gus} gene, confirmed the efficient integration of gene which is also evident with the PCR amplification of both \textit{gus} and selectable marker \textit{hptII} gene. Generally, in microprojectile bombardment mediated genetic transformation, multiple copies of inserted genes are reported (Altpeter et al., 2005), however, in this work, mostly (75%) single copy of insertion was observed by southern analysis, confirming the efficacy of the present method.

4.3.2.2 Microprojectile bombardment mediated transformation with \textit{SbNHX1} gene

Successful realization of optimized genetic transformation protocol for introgression of desired gene of interest is the ultimate objective for obtaining genetically improved transgenic plants. Optimized microprojectile bombardment protocol (Joshi et al., 2011) was applied for bombardment of \textit{Jatropha} embryo axes with recombinant binary plasmid construct pCAMBIA1301-\textit{SbNHX1}. \textit{SbNHX1}-transgenic plants were generated with 38.17% transformation efficiency and 2.12% overall regeneration efficiency (Table 3.10, A7). Transgenic line JL2 showed single copy gene integration while JL8 showed
three copies. The outcome of southern hybridization of transgenic lines performed during protocol optimization and RTqPCR of SbNHX1-transgenic lines showed that single copy gene insertion is achievable by microprojectile bombardment with optimized parameters. To the best of our knowledge, this is the first study for trait improvement in J. curcas via microprojectile bombardment.

4.4 Comparison between transformation methods

Agrobacterium mediated transformation is the most widely used method due to its single copy gene insertion, simple pattern, lower cost and efficient transformation in many dicot plants with extension of its host range to various monocots and other recalcitrant crops (Gelvin 2003b). Particle bombardment is preferred physical transformation method over others physical methods because of its wide range of host including diverse types of cells to be transformed and other applications (Alpeter et al., 2005; Rivera et al., 2012). Agrobacterium and particle bombardment mediated transformation methods were compared with reference to transformation efficiency, overall regeneration efficiency after transformation and copy number of integrated gene to find a better method for J. curcas genetic transformation.

Explants transformed with Agrobacterium showed better transient gus expression (data not shown) than microprojectile bombardment. However, transformation efficiency for J. curcas after hygromycin selection was found 38.17% for microprojectile bombardment that is higher than Agrobacterium mediated transformation (34.81%) using embryo axis as explant. Similarly, overall regeneration efficiency was 2.12% for microprojectile bombardment compared to 1.62% observed for Agrobacterium mediated transformation using embryo axis explants (Table 3.10). Though transient gus expression via Agrobacterium is higher than microprojectile
bombardment, there might be some possible reasons for its lower transformation and overall regeneration efficiency.

- First, even though it is reported that cefotaxime does not adversely affect the growth of transformed cells and plant regeneration in *Jatropha* (Li et al., 2008b; Kumar et al., 2010), but somehow it slows down the growth of explants. Similar results were observed by Zong et al. (2010) in *J. curcas* young leaves and Valvekens et al., (1988) from root explant of *Arabidopsis*.

- Second, *Agrobacterium* cells impose additional stress to the growth of plant cells and cell death following *Agrobacterium* infection still remains a significant limitation (Gelvin 2003b).

- Third, *J. curcas* is considered as a recalcitrant crop which does not easily respond to tissue culture and *Agrobacterium* mediated transformation due to secretion of latex and various phenolic compounds from the wound sites of leaf explants. This inhibits the proper attachment of bacterial cells to host tissue and hence negatively affects transformation efficiency.

Further, contrary to belief that microprojectile bombardment method produces transgenic plants with multiple copies is not the case for *J. curcas*, although the frequency of single gene integration may be higher for *Agrobacterium* method that needs to be studied further. While comparing the rooting efficiency, it was observed that wild type plants showed higher root induction efficiency than transgenic plants that might be possible due to hygromycin selection stress for microprojectile bombardment and other additional stresses for *Agrobacterium* mediated transformation.

Similar to this study, other researchers have simultaneously used both the transformation methods and compared the advantages and disadvantages of these methods for the particular plant species *i.e.* rice (Dai et al., 2001), maize (Shou et al,
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2004), soybean (Li et al., 2004), barley (Travella et al., 2005), Agave (Flores-Benítez et al., 2005), St. John’s wort (Franklin et al., 2005), Tall fescue (Gao et al., 2008), sugarcane (Jackson et al., 2012) etc.

4.5 Physiological analysis

Physiological analyses of transgenic plants represent the extent of expression of integrated gene responsible for a trait. Response of SbNHX1-transgenic lines JL2 and JL8 was assessed with reference to wild type plants. In the leaf disc assay of T0 plants, we studied the effect of NaCl on WT and transgenic lines in dose-dependent manner. Transgenic lines JL2 and JL8 showed better response than WT and chlorophyll content was found higher at 100 and 200 mM NaCl concentrations after 8 days. However at 50 mM NaCl, there was no visible adverse effect on WT in comparison of transgenic lines and chlorophyll amount was at par with 0 mM NaCl concentration. Results show the positive effect of Na\(^+\)/H\(^+\) antiporter SbNHX1 in T0 plants and its enhanced salt tolerance. Similarly, transgenic tobacco plants overexpressed with SbNHX1 showed better growth and chlorophyll content than WT plants at 100-300 mM NaCl concentrations (Jha et al., 2011). There are many reports showing the enhanced salt tolerance in many plant species by overexpression of NHX1 isoforms from different source plants (Rodríguez-Rosales et al., 2009). Recently, Liu et al. (2012) isolated KcNHX1 and KcNHX2 genes from halophyte Karelinia caspica and showed that RNAi silencing of KcNHX1 reduced salt tolerance of K. caspica plants at 200 mM NaCl in leaf disc assay and chlorophyll content was decreased. Gene PgNHX1 from Pennisetum glaucum has been reported to confer high level of salinity tolerance when overexpressed in Brassica juncea. Transgenic plants could withstand higher salt stress than WT in leaf disc assay and in pot (Rajagopal et al., 2007). Previously, overexpression of AtNHX1 in many plant species i.e. tomato (Zhang and Blumwald, 2001), rapeseed (Zhang et al.,
2001), wheat (Xue et al., 2004), tall fescue (Zhao et al., 2007b) and buckwheat (Chen et al., 2008) showed enhanced salt tolerance.

In this study, transgenic J. curcas plants overexpressing SbHX1 were produced via Agrobacterium and microprojectile bombardment mediated transformation. A number of T0 transgenic plants are growing in the green house and getting seeds, salt tolerance level of true-to-type T1 transgenic plants needs to be studied further.