

CHAPTER-II

REVIEW OF LITERATURE

Cultivation and improvement of crops have been the key to agriculture and civilization. Cultivation of *J.curcas* on degraded soils will not only help in their re-vegetation but also help to reduce dependency on import of crude oil. However, large scale cultivation of *J.curcas* remains the most critically important issue that will ultimately decide the success. The major limitation in large scale cultivation as an energy crop is the low and inconsistent seed yield, especially under unattended conditions as might be realistically expected for large scale cultivation on wasteland. Besides improved germplasm, optimum agricultural practices that are practically reliable on large scale on wasteland is one of the real challenges because the species grow in areas with extreme climates and soil conditions that could not be inhabited by most of the agriculturally important plant species (Francis et al., 2005). *J. curcas* cultivation is not only good for using wasteland but it is also renewable source of biodiesel (Takeda, 1982; Jones and Miller, 1991; Openshaw, 2000; Mandpe et al., 2005).

Improvement of this important plant is now a major target for scientist. Two major approaches i.e, agricultural practices and biotechnological approach have been suggested. The agricultural practices are to use advance soil management and irrigation technology. But these practices are time consuming and labour intensive. This shows that agricultural practices are difficult in the near future and hence, biotechnological approach to the problem is more practical. The biotechnological approach to the problem is to select and improve the species by the micropropagation of high yielding genotypes or by introgression of more desirable agronomic traits. Biotechnological crop improvement thus, appears to be the only time effective, alternative approach wherein, transgenic production will be the most important in achieving the above parameters. But for the transgenic approach, efficient plant regeneration protocol from from isolated plant cells or tissue is prerequisite. Recombinant DNA

technology and tissue culture, together with the recent gene transfer methods like biolistic, electroporation, micro-injection, Poly ethylene glycol, silicon carbide fibre and liposomes now enable to target gene into plants even from distantly related organism like bacteria, virus, animals and even humans (Crossway et al., 1986; Fraley, 1986; Fromm et al., 1987; De la pena et al., 1987; Klein et al., 1987; Kaepler et al., 1990; Zhang et al., 1997). Of these, *Agrobacterium*-mediated transformation is preferred method of gene transfer for reasons like simplicity, cost effectiveness, little re-arrangement of transgene, ability to transfer relatively long DNA segments (Hamilton et al., 1997), and preferential integration of foreign genes into transcriptionally active regions (Konez et al., 1989; Ingelbrecht et al., 1991) thereby ensuring proper expression of transgenes in plants (Hernandez et al., 1999) as compared to other methods.

Therefore, *J. curcas* improvement programmes by modern methods of agrobiotechnology are of interest worldwide. This has increased the importance of developing tissue culture methods to facilitate large scale production of true to type plants and for the improvement of the species using genetic engineering techniques. The present review, therefore will briefly but critically discuss recent findings and thoughts in these areas with particular emphasis on regeneration and *Agrobacterium*-mediated genetic transformation.

2.1. *In vitro* micropropagation

A wide range of techniques are available for germplasm conservation and mass multiplication of plant genetic resources. These include seed germination, micropropagation, regeneration from leaf or callus, embryo rescue, micrografting and cryopreservation. *In vitro* techniques offer the possibility of rapid clonal propagation of important plants allowing production of genetically stable true to type progeny (Hu and Wang, 1983; Rani et al., 1995; Sonia et al., 2001; Rout, 2002; Latoo et al., 2006; Qin et al., 2006). Micropropagation not only means mass multiplication of existing stocks of germplasm, but also for the conservation of important, and rare plants that are threatened with extinction (Bajaj, 1991; Tefera and Wannakrairoj, 2004; Gonjalez et al., 2006; Thakur and Karnosky, 2007). The totipotancy of plant cells and the relative ease with which they can be

cultured *in vitro* have generated the optimism that cell and tissue culture can provide, a useful new technology for plant breeders (Davies, 1981).

The origin of plant cell culture date back to beginning of this century when Heberlandt (1902) observed that isolated cells should be capable of developing as “artificial embryos”. Since then, the culture and continued growth of isolated plant tissues and organs in defined media and under standard conditions have been major objectives, important alike to physiologist and morphologists. A considerable measure of success was first achieved with tissues and organ cultures of selected dicotyledonous plants mainly through the pioneer efforts of Robins (1922), Gautheret (1935, 1983, 1985), White (1942, 1954). Wetmore and Morel (1949) using tissues from various cryptogams and also from certain monocotyledonous plants showed that the tissue culture technique is much more generally applicable (Wetmore and Wardlaw, 1951). Later, even leaves (Steeves, 1956), ovules (Maheshwari and Nirmala, 1958), immature fruits (Nitsch, 1951), protoplasts (Cocking, 1960) and anthers (Guha and Maheshwari, 1964) responded to culture treatments. The technological milestones in the history of plant cell and tissue culture, somatic cell genetics, and genetic manipulation of plants and their applications and limitations have been elegantly reviewed and discussed (Murashige, 1978; Bhojwani and Razdan, 1983; Gautheret, 1983, 1985; Cocking 1989; Thorpe, 1990).

2.2. Organogenesis

Basically, there are two modes of organogenesis (i) Direct formation of buds/plantlets from excised explant and (ii) indirect regeneration of plants via callusing/somatic embryos. Direct organogenesis ensures clonal fidelity whereas, indirect organogenesis often shows genetic erosion. The regulation of organogenesis *in vitro* can be achieved by different types of manipulation. These are selection of inoculum (explant), plant growth regulators, proper choice of the culture medium, strength and combination of medium, source, orientation of explant, genotype, type and concentration of source carbohydrate and culture condition (Huetteman and Preece, 1993; Lin et al., 1997; Feyissa et al., 2005; Chitra and Padmaja 2005; Deore and Johnson 2008; Reddy et al., 2008).

2.2.1. Factors affecting organogenesis

2.2.1.1. Media

Significant effect of media has been observed on plant regeneration from different parts of plant (Sharswat and Chand, 2004). Various basal media like White medium, Nitsch and Nitsch medium, B5 medium and Gamborg medium for micropropagation (Khan et al., 1988; Prakash and Gurumurthi, 2004; Diallo et al., 2008), have been employed, but most widely used culture medium is Murashige and Skoog (1962) (MS medium), because most of the plants respond favorably to MS medium, since it contains all the nutrients essential for plant growth *in vitro*. Selection, strength and combination of media are also one of important parameter for optimizing the regeneration protocol (Khan et al., 1988; Zukar et al., 1997; Prakash and Gurumurthi 2005; Diallo et al., 2008).

2.2.1.2. Plant growth regulators

Plant growth regulators are organic compounds naturally synthesized in plants and influence growth and development. There are several classes of plant growth regulators ie. Cytokinins, auxins, gibberellins, ethylene and abscisic acid are available. The mode of differentiation, i.e. organogenesis, embryogenesis, and rhizogenesis, is controlled by the type, concentration and combinations of plant growth regulators like Auxins (IBA, IAA, 2,4-D and NAA), Cytokinins (BAP, TDZ, Zeatin and Kinetin) and its combination (Reinert et al., 1977; Huetteman and Preece, 1993; Lin et al., 1997; Feyissa et al., 2005; Chitra and Padmaja, 2005; Deore and Johnson, 2008; Reddy et al., 2008). Higher concentration of auxins generally induces undesirable callusing, whereas, cytokinins regulate shoot bud formation. When cytokinins are incorporated in to media for micropropagation, high cytokinin concentration produced within shoots, stimulates continued growth and also inhibits apical dominance resulting in a branched axis (Huetteman and Preece, 1993; Lin et al., 1997; Feyissa et al., 2005; Chitra and Padmaja, 2005; Deore and Johnson, 2008; Reddy et al., 2008).

2.2.1.3. Nutrients and vitamins

Nutrients and vitamins are the two major considerations for the medium preparation when optimizing the tissue culture protocol. Shoot formation can be optimized by manipulation of medium phosphate and nitrogen level (Yang et al.,

1995). George and Sherrington (1984) critically reviewed the effect of micronutrient on plant tissue culture. Asamo et al. (1996) found that thiamine and riboflavin favoured induction of embryogenic callus from seed of *Zoysia japonica*. Kintzios et al. (2000, 2001) reported on the effect of micronutrients and vitamins significantly promote callus growth and somatic embryogenesis in rose and chilli pepper.

2.2.1.4. Energy source

Sucrose is by far the most used energy source, for several reasons. It is cheap, readily available, relatively stable to autoclaving, and readily assimilated by plants. Other carbohydrates can also be used, such as glucose, maltose and galactose as well as the sugar-alcohols glycerol and sorbitol (Fowler, 2000). The concentration and type of energy source in culture medium plays an important role in shoot multiplication and elongation (Dodds and Roberts, 1985; Gurel and Gulsen, 1998; Dorion et al., 2004; Reddy et al., 2008). Gurel and Gulsen (1998) observed during the proliferation and transplantation stages, a steady increase in the mean shoot production and mean shoot growth rate per explant was observed with increasing concentration of sucrose (5 and 6%) which was used as a energy source. This may be mainly due to the fact that high sugar levels available in the culture medium may speed up cell division thus leading to an increase in the volume and weight of tissues cultured, as suggested by Chong and Taper (1972). Rugini (1984) who suggested that 3% sucrose should be used in all culture stages for the *in vitro* multiplication of almond. The stimulating effect of sucrose on shoot or plant regeneration has also been shown in lily species (Gerrits and De Klerk, 1992; Bonnier and Van Tuyl, 1997). Nhut et al. (2001) showed that 1 to 2% sucrose promoted the regeneration of shoots, whereas, 3 to 4% increase shooting and limited rooting.

2.2.1.5. Type of explant

Type of explant is also one of the important factors in optimizing the tissue culture protocol. Type of explants like leaf, petiole, cotyledonary leaf, hypocotyle, epicotyle, embryo, internode and root explant significantly effect on tissue culture process of plants (Khan et al., 1988; Sujatha and Mukta, 1996; Tyagi et al., 2001; Gubis et al., 2003; Alagumanian et al., 2004; Ali and Mirza, 2006). This may be

due to the different level of endogenous plant hormones present in the plants parts. Leaf is the most commonly used explant for regeneration due to more surface area available (Sujatha and Muktha, 1996; Tyagi et al., 2001). Tyagi et al. (2001) used root, shoot, and leaf explant and maximum regeneration efficiency was observed from leaf explants in *Cajanus cajan*. Sujatha and Mukta (1996) used different explant like leaf, petiole, hypocotyle and maximum regeneration frequency was observed from leaf explant of *Jatropha curcas*. Alagumanian et al. (2004) used leaf and stem explant and maximum regeneration efficiency observed from stem explant in *Solanum trilobotam*. Gubis et al. (2003) used hypocotyle, epicotyle, cotyledons, leaf, petiole, internode and maximum response were obtained from hypocotyle in Tomato. Ali and Mirza (2006) used root, stem, leaf and petiole but maximum responses were observed from stem explant in *Citrus jambhiri* Lush.

2.2.1.6. Genotype

Genotype is also one of the most important factors affecting regeneration (Tyagi et al., 2001; Gubis et al., 2003; Gandonou et al., 2005; Feyissa et al., 2005; Chitra and Padmaja, 2005; Landi and Mezzeti, 2006; Reddy et al., 2008). Genotypic effect on shoot regeneration and elongation has 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.

2.2.1.7. Source of explant

Source of explant i.e. *in vitro* and *in vivo* is also important for regeneration (Reddy et al., 2008). *In vitro* explant is considered to be the most suitable for organogenesis (Reddy et al., 2008). The fact that source of explant has different capacity of regeneration are well documented (Feyissa et al., 2005). *In vitro* explant in general has better potential to organogenesis as compared to *in vivo* explant (Reddy et al., 2008). The difference may be due to the level of endogenous hormones present in the plant explant. Seedling explant is more

responsive or meristematic than mature plants (Teng, 1999; Feyissa et al., 2005) due to different level of plant hormones present in the plants.

2.2.1.8. Orientation of explant

Orientation of explant in the culture medium also affects the regeneration efficiency (Sharma and wakhlu, 2001; Arockiasamy et al., 2002). In general regeneration efficiency is higher in horizontal position as compared to vertical condition of explant due to little contact of explant to medium in vertical position as compared to horizontal position. The initiation site, polarity, and efficiency of bud regeneration were altered by explant orientation is well documented in *Dionaea muscipula* (Teng, 1999). Cotyledons placed in abaxial (lower surface facing down) orientation consistently produced better shoot regenerative response and produced greater numbers and taller shoots compared to those inoculated in adaxial (upper surface facing down) orientation (Bhatia et al., 2004, 2005).

2.2.1.9. Culture condition

Culture condition is also one of the important parameter in optimization of tissue culture protocol. Culture condition includes light intensity, duration of photoperiod and temperature considerably effect regeneration (Welandar, 1988; Pooler and Scorza, 1995; Miguel et al., 1996; Teng et al., 1999; Gentile et al., 2002, 2003). Zalunskite et al. (2007) observed that regeneration frequency increased when cultures were maintained in the dark for 4 weeks and then transferred to 16 h photoperiod for 4 weeks. The dark period may be important because of its possible influence on endogenous hormones of the levels and interaction with exogenously applied growth regulators that may promote adventitious shoot regeneration. Temperature influence on various physiological processes, such as respiration and photosynthesis, is well known and it is not surprising that it profoundly influences plant tissue culture and micropropagation. The most common culture temperature range is between 20°C and 27°C, but optimal temperatures vary widely, depending on genotype (Altman, 2000; Read and Preece, 2003).

2.3. Root induction and acclimatization

There is a high mortality rate due to failure of root induction in *in vitro* shoots or subsequent problems in acclimatization. The adventitious root induction in relation to micropropagation has great relevance and is being discussed and reviewed by many workers (Nemeth, 1986; Moncousin, 1987; Wilson and Van Staden, 1990; Haissing et al., 1992; Sujatha and Mukta, 1996; Vuylasteker et al., 1998; Ahmed et al., 2006; Nandagopal and Kumari 2007; Singh et al., 2008; Reddy et al., 2008). To induce rooting the explant is often grown aseptically in a medium containing auxins such as IAA, IBA, or NAA (Sujatha and Mukta, 1996; Vuylasteker et al., 1998; Ahmed et al., 2006; Nandagopal and Kumari, 2007; Singh et al., 2008; Reddy et al., 2008), however, some workers developed a simple method of inserting *in vitro* shoots directly in to sand/soil (Rajbhandary and Baja, 1991; Mereti et al., 2002; Arya et al., 2001). A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environment. So, transplantation stage continues to be a major bottleneck in the micropropagation of many plants (Hazarika, 2003). The benefit of any micropropagation system can however, be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro*.

2.4. Genetic transformation

Though conventional plant breeding methods have resulted in a spectacular improvement in crop production, there are strong pressures for further improvement in crop quality and quantity due to explosion in population, social demands, health requirements, environmental stresses and ecological considerations (Kung, 1993). Conventional plant breeding techniques have limitations as these depend on sexual compatibility and often take 10-15 years to release a new variety due to extensive backcrossing (Pauls, 1995). These limitations have stimulated the development of more advanced technologies like genetic transformation of plants. Genetic transformation can be defined as the transfer of foreign genes isolated from plants, viruses, bacteria or animals into a new genetic background. In plants, successful genetic transformation requires the production of normal, fertile plants, which express the newly inserted genes.

The process of genetic transformation involves several distinct stages, namely insertion, integration, expression and inheritance of the new DNA. Process of gene insertion can involve the use of bacterial (*Agrobacterium* species) or viral vectors or direct gene transfer methods (Webb and Morris, 1994). Genetic engineering has allowed explosive expansion of our understanding in the field of plant biology and provides us with the technology to modify and improve crop plants. A remarkable progress has been made in the development of gene transfer technologies (Gasser and Fraley, 1989) which ultimately have resulted in production of a large number of transgenic plants both in dicots and monocots. Potential benefits from these transgenic plants include higher yield, enhanced nutritional values, reduction in pesticides and fertilizer use and improved control of soil and water pollutants. Some of the important characters like resistance to herbicide (Smith, 1994), disease (Smith, 1994), insect (Perlak et al., 1990), high protein content (Habben and Larkins, 1995), cold tolerance (Georges et al. 1990), fruit quality (Fray and Grierson, 1993), biodegradable plastics (Poirier et al., 1995), antibodies and vaccines (Mason et al., 1992) etc. have been incorporated in the genetically engineered plants.

2.4.1. History of genetic transformation

It took more than 2000 years to detect the principle cause of the crown gall disease after it was first described by Aristotle's and Theophrastus (Siemens and Schieder, 1996). Smith and Townsend (1907) were the first to report that *Agrobacterium tumefaciens* is the causative agent of the widespread neoplastic plant disease crown gall. Since then a large number of scientists throughout the world have focused their research to understand the molecular mechanism of crown gall induction. The soil bacterium *A. tumefaciens* and *A. rhizogenes* are considered as natural genetic engineers due to their ability to transfer and integrate DNA into plant genomes through a unique integrative gene transfer mechanism (Jouanin et al., 1993). It was only in 1983 that scientist inserted the first foreign genes into *Petunia* and tobacco (Kung, 1984). *Agrobacterium*-mediated gene transfer became the method of choice due to convenience and high probability of single copy integration. Independently, several transgenic tobacco plants were produced to express foreign genes

engineered by the *Agrobacterium tumefaciens* vectors (Murai et al., 1983; Horsch et al., 1984; De Block et al., 1984). Initially successes in genetic transformation were limited to the species of *Solanaceae* especially tobacco (*N. glauca* L.). However, this changed the situation dramatically in late 80's and early 90's and resulted in transformation of a wide range of plants for agronomically important traits using genetically engineered avirulent strains of *Agrobacterium* as vectors (Herrera-Estrella et al., 1983). Since the initial successes in the *Agrobacterium*-mediated transformation were mostly confined to dicotyledonous plants, concerted efforts were made to look for alternative methods of gene transfer. Method of direct gene delivery into protoplasts was the next development in genetic transformation (Draper, 1982), Further many more techniques such as macroinjection (Zhou et al., 1983), soaking pollen in DNA solution (Ohta, 1986), pollen transformation via pollentube pathway (Luo and Wu, 1988), microinjection (Neuhaus and Spangenberg, 1990), silicone carbide fibres (Kaepler et al., 1990), electroporation (Dekeyser et al., 1990), sonication (Joersbo and Brunstedt, 1990), electrophoresis (Griesbach & Hammond, 1993), laser mediated gene transfer (Guo et al. 1995) have been developed. However, none of these approaches has, so far, been developed into a reproducible universal gene transfer technique (Potrykus, 1990). The next breakthrough in genetic transformation was the development of biolistic (Particle bombardment) transformation approach (Klein et al., 1987; Sanford 1988).

2.4.2. *Agrobacterium*-mediated gene transfer

Agrobacterium is a gram-negative, soil-dwelling bacterium, which infects plant cells near wounds, usually at the junction between the root and stem (crown) in a wide range of plant species. *Agrobacterium*-mediated gene transfer involves incubation of cells or tissues with the bacterium (cocultivation), followed by regeneration of plants from the transformed cells. For plant species that are readily amenable to tissue culture, *Agrobacterium*-mediated gene transfer, the first widely adopted methods of developing transgenic plants, remains the most popular technique. Probably the greatest advantage of the system is that it offers the potential to generate transgenic cells at relatively high frequency, without a significant reduction in plant regeneration rates. The system is simple,

inexpensive and in many cases efficient. Moreover the DNA transferred to the plant genome is defined, it does not normally undergo any major rearrangements and it integrates into the genome as a single copy (Walden and Wingender, 1995). *Agrobacterium tumefaciens* possess a tumor inducing (Ti) plasmid responsible for the tumor formation (Zupan and Zambryski, 1995) whereas, *A. rhizogenes* possess a root inducing (Ri) plasmid which is responsible for DNA transfer and the resulting hairy root formation (Bevan and Chilton, 1982; Tepfer, 1984). During infection, the bacterium transfers a small section of its own genetic material (TDNA) into the genome of the host plant's cell (Zambryski, 1992). Once inserted, the bacterial genes are expressed by infected cells of that plant. During the infection process, first the plant cell begins to proliferate and form tumors and then synthesize an arginine derivative called opine. The opine synthesized is usually nopaline or octopine depending on the strain involved. These opine are catabolized and used as energy sources by the infecting bacteria. By understanding and manipulating this process of infection or transformation, scientists have been able to harness these powerful and sophisticated vectors to transfer specific cloned genes of major importance. Initially, monocotyledons were considered outside the host range of *Agrobacterium*. However, advances in understanding of the biology of the infection process, availability of gene promoters suitable to monocotyledons (Wilmink et al., 1995) as well as selectable markers have improved transformation of monocotyledons (Smith and Hood, 1995). Transgenic plants of Citrus (Moore et al., 1992), rice (Hiei et al., 1994) and maize (Ritchie et al., 1993) have been produced via *Agrobacterium*-mediated transformation. However, success of *Agrobacterium*-mediated transformation depends on the cultivar (Robinson and Firoozabady, 1993), the choice of explant (Robinson and Firoozabady 1993; Jenes et al., 1993) the delivery system, the *Agrobacterium* strain (Gelvin and Liu, 1994); the conditions of cocultivation, the selection method and the mode of plant regeneration. *Agrobacterium* cocultivation has been successfully used for the transformation of leaves, roots, hypocotyls, petioles, cotyledons (Binns and Thomashow, 1988; Zambryski, 1992; Hooykaas and Beijersbergen, 1994), pollen-derived embryos (Sangwan et al., 1991, 1993), seeds (Feldmann and Marks, 1987) and even

plants (Cheng et al., 1997). T-DNA of *Agrobacterium* is a small section of the plasmid DNA, about 23 kb in size, which makes up about 10% of the Ti or Ri plasmids. This stretch of DNA is flanked by 25bp repeated sequences, which are recognized by the endonucleases encoded by the vir genes. Within the T-DNA, two distinct regions TL and TR have been identified. The T-DNA of nopaline strains can integrate as a single segment, whereas octopine strains frequently integrate as two segments TL and TR. TL carries the genes controlling auxin and cytokinin biosynthesis and is always present when tumors are formed. Failures of TR to integrate results in the loss of opine biosynthesis (Webb and Morris, 1992). The vir (virulence) region of Ti plasmid contains the genes which mediate the process of T-DNA transfer. Vir gene action generates and processes a T-DNA copy and facilitates T-DNA movement out of the bacterium and into the plant cell. Helper plasmids for non-oncogenic plant transformation have been developed to utilize the vir gene functions with T-DNAs containing genes of choice (Hood et al. 1993). The removal of the oncogenes from the Ti plasmid results in disarmed strains of *A. tumefaciens* (Klee et al., 1987). The oncogenes of *Agrobacterium* are replaced by reporter genes/screenable marker genes (e.g. b-glucuronidase gene (*gus*), luciferase (*luc*) gene for analyzing gene expression. Genes conferring resistance to antibiotics (e.g. neomycin phosphotransferase II (*nptII*), hygromycin phosphotransferase (*hpt*), phosphinothricin acetyl transferase (*bar*) are used to allow selection between transgenic and non transgenic cells. Also oncogenes have been replaced by genes of economic importance (McElroy and Brettel, 1994). Plants are usually transformed with relatively simple constructs, in which the gene of interest is coupled to a promoter of plant, viral or bacterial origin. Some promoters confer constitutive expression while others may be selected to permit tissue specific expression. The cauliflower mosaic virus (CaMV) 35S RNA promoter is often used because it directs high levels of expression in most plant tissues (Walden and Wingender, 1995).

2.4.3. Mechanism of *Agrobacterium* infection, T-DNA transfer and integration

Plant species differ greatly in their susceptibility to infection by *Agrobacterium tumefaciens* or *rhizogenes*. Even within a species, different

cultivars or ecotypes may show different degree of susceptibility. These differences have been noted in a variety of plant species. The subject matter has been reviewed (Krens et al., 1985; Gelvin, 2000). Though environmental or physiological factors are attributed for these differences, genetic basis for susceptibility has recently been described in *Arabidopsis* (Nam et al., 1997). *Agrobacterium* attaches to plant cells in a polar manner in a two-step process. The first step is likely mediated by a cell-associated acetylated, acidic capsular polysaccharide (Reuhs et al., 1997). The second step involves the elaboration of cellulose fibrils by the bacterium, which enmeshes large numbers of bacteria at the wound surface (Matthysse et al., 1982). The interaction between *Agrobacterium* spp. and plant involves a complex series of chemical signals communicated between the pathogen and the host cells. These signals include neutral and acidic sugars, phenolic compounds, opines (crown gall specific molecules synthesized by transformed plants), Vir (virulence) proteins and the T-DNA (Gelvin, 2000). Baker et al. (1997) has described the chemical signaling in plant-microbe interactions. The T DNA transfer process initiates when *Agrobacterium* perceives certain phenolic compounds from wounded plant cells (Hooykass and Beijersbergen, 1994) which serves as inducers or coinducers of the bacterial *vir* genes. Phenolic chemicals such as acetosyringone and related compounds (Dye et al., 1997) are perceived via the VirA sensory proteins (Doty et al., 1996). Most of the induced Vir proteins are directly involved in T-DNA processing from the Ti plasmid and the subsequent transfer of T-DNA from the bacterium to plant. Among them VirD2 and VirE2 contain plant active nuclear localization signal sequences (NLS) (Herrera-Estrella et al. 1990). VirD2 protein is directly involved in processing the T-DNA from the Ti plasmid. It nicks the Ti plasmid at 25-bp directly repeated sequences, called T-DNA borders that flank the TDNA (Filichkin and Gelvin, 1993; Veluthambi et al., 1988, 2003). Thereafter, it strongly associates with 5' end of the resulting DNA molecule (Filichkin and Gelvin, 1993) through tyrosin (Vogel and Das, 1992). VirD2 contains two nuclear localization signal (NLS) sequences (Herrera-estrella et al., 1990) whereas VirE2 contains two separate bipartite nuclear localization signal (NLS) regions that can target linked reporter proteins to plant cell nuclei (Citovsky et al., 1994). A model

for *Agrobacterium*-mediated genetic transformation is represented in plate 1. Many plant species are still recalcitrant to *Agrobacterium* transformation. This recalcitrance does not result from a lack of T-DNA transfer or nuclear targeting, rather its integration into the genome of regenerable cells appears to be limiting. In the future, it may be possible to overexpress endogenous genes involved in the integration process or to introduce homologous genes from other species, and thereby affect higher rates of stable transformation (Gelvin, 2000).

2.4.4. Factors affecting *Agrobacterium* infection and transformation efficiency

Ever since the first genetically engineered *Agrobacterium* was used to produce a transgenic plant (Holford et al., 1992; Hooykaas and Schilperoort, 1992; Mantis et al., 1992; Sheng and Citovsky, 1996; Wei et al., 2000; Zupan et al., 2000), a wide variety of plants have been genetically modified for crop improvement and many of them have been commercialized. However, production of such transgenic plants involves the modification of a number of parameters due to the affinity of *Agrobacterium* to specific host plants only. The different factors that have been optimized are discussed below.

2.4.4.1. Bacterial strain / vector

The fact that different strains have different capacity of transform tissues or plants are well documented (Higgins, 1992). The nopaline strains in general have better potential to infect woody species as compared to the octopine ones (Ahuja, 1987). The difference may be due to the lack of “overdrive” sequences in the commonly used binary vectors that are derived from pBin19. Overdrive sequence is more essential for octopine strains than the nopaline ones. The other differences may be due to the chromosomal virulence genes (chvs) which are related to the attachment of *Agrobacterium* to the plant cell walls. The octopine strains are specifically characterized by the virF gene, or a host range determinant that is induced by acetosyringone (Jarchow et al., 1991). The nopaline strains are more effective than the octopine strains and have been demonstrated in case of grapes wherein the GV3101 strain was more superior (Berres et al., 1992). The strains play a significant role in transformation efficiency has been further proved in the Novel Orange *Citrus sinensis* (Bond and

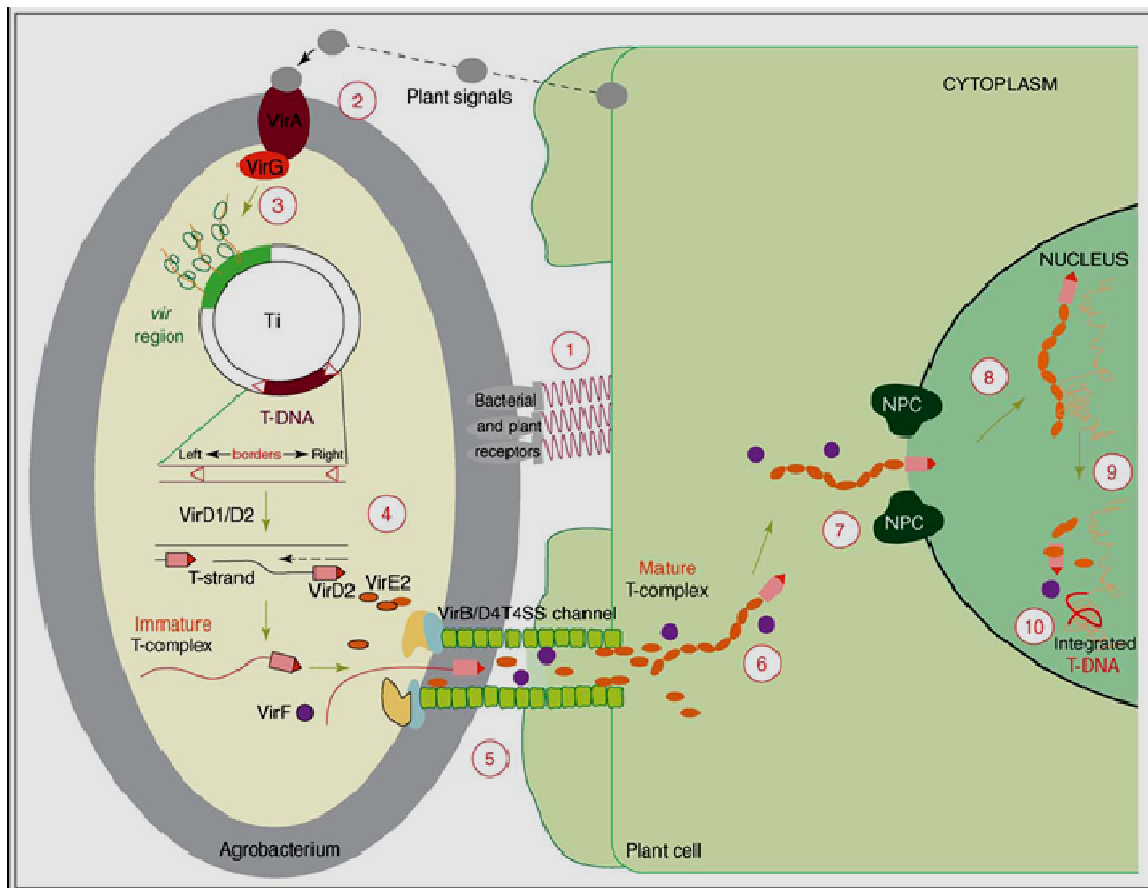


Plate 1. A model for the *Agrobacterium*-mediated genetic transformation.

The transformation process comprises 10 major steps and begins with (1) recognition and attachment of the *Agrobacterium* to the host cells (2) sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system. (3) activation of the vir gene region (4) a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (5) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (6) Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (7) and is actively imported into the host-cell nucleus (8) once inside the nucleus, the T-DNA is recruited to the point of integration (9) stripped of its escorting proteins (10) integrated into the host genome.

Roose, 1988). Bacterial strains and vectors are known to affect transformation efficiency of plants. Thus, when Hiei et al. (1994) tested different combinations of two strains and three binary vectors in rice, only the strain LBA 4404 (pTOK233) was the most efficient. Surprisingly, the combination of the super virulent strain EHA101 and the super-binary vector pIG121Hm were less efficient than the LBA4404 (pIG12Hm) alone. Hamilton et al. (1997) also showed that *Agrobacterium* could transfer DNA fragments as large as 150 kb into the plant genome by employing the principle of bacterial artificial chromosome (BAC) into the binary vectors, thereby generating the so-called binary BAC (BiBAC). Veluthambi et al. (2003) reviewed the use of new series of vectors like the small and stable pZPs, the pCAMBIAs with single cloning sites and the pART series with multiple cloning sites.

2.4.4.2. Pre-culture/wounding

Transformation efficiency is also considerably affected by pre-culturing and co-cultivation period (Hiei et al., 1994; Dong et al., 1996; Ishida et al., 1996; Rashid et al., 1996; Cheng et al., 1997). Pre-culturing induces cell division in explant and makes them more receptive to *Agrobacterium* and is largely dependent on the time of pre-culture. While two days of pre-culture was required for the leaf discs of quaking aspen (*Populus tremuloides*), the time period required for pre-culture was genotype dependent in case of almonds (Tsi et al., 1994). However, negative effect of pre-culture on woody plant transformation was also observed in almonds. Pre-culturing of leaf pieces for two days was found to reduce the transformation efficiency to 10 % in *Cyphomandra betacea* (Altkinsons and Gardner, 1993), and tea (Mondal et al., 2002) was also observed when explant was not pre-cultured.

2.4.4.3. Bacterial density and growth phase

Agrobacterium cell density, as well as the stage of bacterial is also important for genetic transformation (Mathysse, 1986). The late log phase is considered to be the most suitable for transformation in a majority of plants (Mondal, 1999). However, at a high density regeneration of plant tissue is generally inhibited by bacterial-induced stress and controlling the overgrowth of bacteria during co-cultivation becomes difficult. In citrus, the bacterial density of

4×10^7 cells/ml as compared to 4×10^8 cells/ml yielded the maximum (20.6 %) transformation efficiency (Pena et al., 1995). Moreover, O.D. values higher than 0.6 at $A_{600\text{nm}}$ indicating the late log phase were not desirable for the co-cultivation of almond leaf discs (Archilletti et al., 1995). Transformation efficiency in black poplar (Confalonieri et al., 1995) and grapevine (Baribault et al., 1990) however, was not affected by bacterial density.

2.4.4.4. Inducers of vir genes

Several stimuli that are known to induce the vir genes includes - low concentrations of phenolic compounds acetosyringone, hydroxyl-acetosyringone (Stachel et al., 1986; Sheng and Citovsky, 1996), pH (> 5.7) of the medium (Bolton et al., 1986), carbon source as sucrose and glucose (Alt-Moerbe et al., 1988; Hiei et al., 1994; Seo et al., 2002), culture conditions during co-cultivation like temperature (Stachel et al., 1986), darkness and osmoticum (Shimoda et al., 1990; Goodvin et al., 1991; and James et al., 1993, Koichi et al., 2002). However, some reports showed that light rather than dark and temperature as low as 22°C or even lower were crucial to higher transformation efficiency (Zambre et al., 2003). Acetosyringone is known to improve the transformation efficiency in a large number of plant species (Song et al., 1990; Godwin et al., 1991) Acetosyringone has been found to be effective at a wide range of concentrations (20 μM -100 μM) in a number of plant species. Even in the two different cultivars of the same species of grapevine, different concentrations were required i.e. 20 μM (Baribault et al., 1990) and 100 μM (Colby et al., 1991). While 20 μM acetosyringone was effective for peaches (Smigocki and Hammerschlag, 1991), 100 μM was required for trifoliolate oranges (Hiramatsu-Kaneyoshi et al., 1994) and 200 μM enhanced the transformation efficiency of hybrid poplar cv. NC-5339 (Howe et al., 1994). However, acetosyringone failed to bring about transformation in cultivars *Populus deltoids* and *Populus euranericana* (Confalonieri et al., 1994) and tea (Mondal et al., 2001; Kumar, 2003; Kumar et al., 2004). The other important inducer 'glucose' has been reported to bring about transformation in apples (James et al., 1993) and strawberries (Shimoda et al., 1990). Although plant growth regulators have not been considered to be inducers, yet they have been reported to enhance the transformation efficiency in

woody plants when used in the co-cultivation medium (Bondt et al., 1996). The presence of TDZ and NAA in the co-cultivation medium enhanced the recovery of transformed subterranean clover shoots probably because the peripheral cells at the cut surface of hypocotyl responded better when grown on a regeneration medium supplemented with TDZ (Sangwan et al., 1991). Similarly, 2, 4-D was also used in the co-cultivation medium during genetic transformation of tea (Sandal, 2003).

2.4.4.5. Plant variety and explant

The limited host range specificity of *Agrobacterium* is a well documented fact (Nester et al., 1984; Pueppke et al., 1984; Hawes et al., 1989). *Agrobacterium* has been reported to infect 643 host plants from 331 genera (DeCleene and DeLey, 1976). Anderson and Moore assayed 176 strains of *Agrobacterium* for pathogenicity on 11 dicotyledenous plants and found extensive host range variations between widely amongst the different cultivars or genotypes (Hawes et al., 1989). Different varieties of a single species were also found to respond differently to a particular bacterial strain. The host range of individual strains of *A. tumefaciens* is determined primarily by the Ti plasmid and can range from few to hundred species (Dandekar et al., 1988). Thus, *Agrobacterium*-mediated transformation is highly specific to plant species and cultivars (Riva et al., 1998; Kumar, 2003) and transformation efficiency may vary even with the same cultivars depending upon the explant (Wei et al., 2000). Although the actual biochemical basis for host range variations in *Agrobacterium* is not clear yet, two distinct regions of the Ti plasmid is now thought to contribute to the overall host specificity of the bacterium (Hooykaas and Schilperoot, 1992). These include loci (vir A) in the virulence region and another locus within the T-DNA. Besides, the T-DNA and the virulent genes, there is a certain undefined host factor that influence specificity to some extent (Hood et al., 1993). However, these factors also mediate susceptibility of plants to infection by recombinant strains and may vary even within different parts of the same plant (Martin et al., 1989). Thus, when the same explant (leaf and petiole) was used for different cultivars 'Meeker', 0.91 % for chilliwack and 8.1 % for 'Canby' etc. (Mathews et al., 1995). Considering the larger surface area for manipulation,

easy availability and maintenance of true to type nature are considered to be attractive as explant for biotechnological crop improvement and have been used extensively.

2.5. *Jatropha* tissue culture and genetic transformation

Starry attempts have been made for tissue culture in *J.curcas* like morphogenesis and regeneration (Sujatha and Mukta 1996; Deore and Johnson 2008; Srivastava and Banerjee, 2008), somatic embryogenesis (Sardana et al., 2000; Jha et al., 2007), regeneration from epicotyl callus (Qin et al., 2004; Rajore and Batra, 2007), multiple shoot proliferation from shoot tip (Rajore and Batra, 2005; Datta et al., 2007) and shoot bud proliferation from axillary nodes and leaf sections of non-toxic (Sujatha et al., 2005). Despite sufficient regeneration systems achieved from different explant of *J.curcas*, the presence of intermediary callus or callus-mediated regeneration is least desired for the production of true to type plants. It is also reported that regeneration in *J.curcas* is highly genotype dependent (da Camara Machado et al., 1996). There is only one report on callus mediated genetic transformation in *J.curcas* (Li et al., 2008).