CHAPTER TWO
REVIEW OF LITERATURE

The present review presented the importance of Biofuel, main role of biotechnology to use renewable resources for Biofuel production, tissue culture, growth regulators for callus induction and plant regeneration from maize embryo, role of tissue culture and Biolistic in transfer COMT- antisense, by particle bombardment to maize callus

2.1. BIOFULES AND BIOTECHNOLOGY

The International Energy Agency's New Policies Scenario projects a world demand of 99 million barrels of crude oil per day by 2035, but peak oil production at 68–69 million bpd (http://www.iea.org/press/pressdetail). Something has to fill this gap, even if there are widely enforced efficiency measures and industrial convulsion that might drive oil consumption down. This is simply a resource demand gap, without factoring in the need to address fundamental issues around greenhouse gas emissions (**Zah, 2007**).

In the long term, other technologies may replace the present petroleum fuel driven motor vehicles, heat and power systems, including fuel cells, direct hydrogen, electric vehicles, inter alia. However, in the short to medium term, a replacement is needed that will help reduce carbon dioxide emissions and extend petroleum fuel reserve life. Biofuels have been subject to much scrutiny over the past decade (**Speight, 2011**).

The use of biotechnology for biofuel production is very attractive. Theoretically biofuel yields can be increased without a comparable increase in the amount of energy needed for production or cultivated land. Significant progress has been made using molecular biology in the past decade to increase the activity of enzymes and the microbes used for biofuel production. However, by many estimates, there is not enough arable land to produce sufficient Biofuel feedstocks to replace more than 15% of transportation fuel using existing technologies (**Gressel, 2008**).

The elevation of using ''renewable resources'' as almost romantic sources of energy has been in headlines and is a new target for investors, but a more jaundiced look is needed. If the pros and cons are not fully discussed, the research and development that needs to be done will not be done. Clearly it is

naive, ignorant of history, or conceited to think that one can efficiently grow species as biofuel crops that have not been domesticated for that purpose, yet the cultivation of species that have not undergone years of breeding let alone millennia of selective domestication is being widely promoted. Biotechnology has the potential to assist in rapidly overcoming many of the short-comings of the species being promoted, especially those characters that are intractable to breeding, where exogenous genes are needed, or where tissue-specific or temporal expression or suppression of endogenous genes would be valuable.

Biofuels are not new. The use of biofuels began first when it was learnt how to ignite biological material in prehistoric times to cook food and allow early primate *Homo sapiens* to move from more tropical and subtropical climes, to more temperate climates by heating their caves and huts. Drying cow manure for use of fuel is still a traditional entry-level job of young brides into the realms of their mothers-in-law in rural India. Throughout the developing world firewood is gathered for cooking. Not to be left out, wood-burning home heating was a craze in parts of the developed world a few years ago as part of a romance with renewable resources.

A century ago, 20% of arable land in temperate Europe and North-America was dedicated to oats, the biofuel that powered the horses, mules, and farm laborers of agriculture, as well as much of urban transport (with concomitant urban manure pollution). In all cases this is highly polluting, especially in the developing world when much of the cooking is performed in a chimney-free environment (with a high incidence of pulmonary diseases). The use of manure depletes the soil of potential minerals and organic matter, and uncontrolled gathering of firewood denudes vast areas.

Biotechnology can also be inserted into the industry and directly affect the economy of a sector. An outstanding example of this is that biotechnology may be applied to dramatically decrease costs in corn ethanol production and improve energy input requirements. There are numerous opportunities to improve important characteristics of the corn plant to decrease the cost of ethanol production **Kemble *et al.,* 2006**).

Among the biotechnology tools, those related to transferring DNA have received special attention, leading to several strategies such as Biolistic or *Agrobacterium tumefaciens* (**Gordon-Kamm W.J *et al*. 1990).**

2.2. MAIZE AND TISSUE CULTURE TO PRODUCING BIOFUELS

Corn grain from these varieties would yield increased glucose levels compared to existing varieties and lead to higher levels of ethanol production from the grain

Maize (*Zea mays* L.) is the third most planted cereal crop after wheat and rice worldwide. Globally it is top ranking cereal in terms of productivity and has worldwide significance as human food, animal feed and fodder as well as source of large number of industrial products. It is used as a raw material for manufacture of large number of industrial products like corn starch and starch-based products, and in fermentation and distillation industries (**Wada *et al.* 2008**).

Maize grain is used as feedstock for many large volume industrial products (e.g. ethanol, biodiesel, poly-lactic acid, sweeteners) and has been demonstrated to be an effective expression system for functional proteins of rokaryotic (**Streatfield *et al*. 2001; Chikwamba *et al*. 2002a; Bailey *et al*. 2004); viral (Streatfield *et al*. 2001) and eukaryotic (Hood *et al*. 1997; Zhong *et al*. 1999; Yang *et al*. 2002).**

Plant cell/tissue culture, also referred to as *in vitro,* axenic, or sterile culture, is an important tool in both basic and applied studies as well as in commercial application (**Thorpe, 1990, 2007 and Stasolla and Thorpe 2011).**

Although Street (1977) recommended a more restricted use of the term, plant tissue culture is generally used for the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro.* Perhaps the earliest step toward plant tissue culture was made by Henri-Louis Duhumel du Monceau in 1756, who, during his pioneering studies on wound-healing in plants, observed callus formation (**Gautheret, 1985**).

Extensive microscopic studies led to the independent and almost simultaneous development of the cell theory by **Schleiden (1838)** this theory holds that the cell is the unit of structure and function in an organism and therefore capable of autonomy. This idea was tested by several researchers, but the work of **Vöchting (1878)** on callus formation and on the limits to divisibility of plant segments was perhaps the most important. He showed that the upper part of a stem segment always produced buds and the lower end callus or roots independent of the size until a very thin segment was reached. He

demonstrated polar development and recognized that it was a function of the cells and their location relative to the cut ends.

Haberlandt (1902) opined that to "my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants have been made. Yet the results of such culture experiments should give some interesting insight to the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within a multicellular whole organism are exposed. He experimented with isolated photosynthetic leaf cells and other functionally differentiated cells and was unsuccessful, but nevertheless he predicted that "one could successfully cultivate artificial embryos from vegetative cells." He thus clearly established the concept of totipotency, and further indicated that "the technique of cultivating isolated plant cells in nutrient solution permits the investigation of important problems from a new experimental approach. **White (1963), Bhojwani and Razdan (1983), and Gautheret (1985).**

During the 1990s and the early twenty-first century continued expansion in the application of *in vitro* technologies to an increasing number of plant species has been observed. Tissue culture techniques are being used with all types of plants, including cereals and grasses (**Vasil & Vasil, 1994**), legumes (**Davey *et al.,* 1994**), vegetable crops (**Reynolds, 1994**), potato (**Jones, 1994**) and other root and tuber crops (**Krikorian, 1994a**), oilseeds (**Palmer & Keller, 1994**), temperate (**Zimmerman & Swartz, 1994**) and tropical (**Grosser, 1994**) fruits, plantation crops (**Krikorian, 1994b**), forest trees (**Harry & Thorpe, 1994**), and, of course, ornamentals (**Debergh, 1994**). As will be seen from these articles, the application of *in vitro* cell technology goes well beyond micro propagation and embraces all the *in vitro* approaches that are relevant or possible for the particular species and the problem(s) being addressed. However, only limited success has been achieved in exploiting somaclonal variation (**Karp, 1994**) or in the regeneration of useful plantlets from mutant cells (**Dix, 1994**); also, the early promise of protoplast technology remains largely unfulfilled (**Feher & Dudits, 1994**).

Many researchers such as **Green and Phillips, (1975); Armstrong and Green, (1985); Hodges *et al.* (1986); Lee and Phillips, (1987) and Shillito *et al.* (1989)** suggested that, the regeneration ability of any plant is influenced by different factors. They showed the type of explant is considered one of the main

factors that attracted the concern of many investigators. Also, immature embryos have been the most widely used explant in many cereals, including maize.

In the previous research on tissue culture in maize immature embryos **Armstrong and Green, (1985)** detected two different types of embryogenic callus i.e. Type I, a compact organized and slow-growing callus and Type II, a soft, friable and fast-growing one characterized by its high regeneration capacity. Those results showed that, production of Type II callus arises at low frequency and only for specific genotypes. Also, regenerability is influenced, to a great extent, by the media composition (**Vain *et al*. 1989a, b, Songstad *et al*. 1991, Bohorova *et al,*. 1995 and Carvalho *et al*. 1997**).

Since tissue cultures are normally used for transformation of maize using particle bombardment or *Agrobacterium tumefaciens* (**Gordon-Kamm *et al*. 1990; Ishida *et al*. 1996**), the maintenance of plant regeneration ability after the selection of the transformed callus, which can take up to 2 mo, is most important. This ability is known to be lost over time with maize cultures as with most other plant species (**Reinert 1973; Lozovaya et al. 2006**).

There have been a number of studies of the differences in basic morphology and biochemical characteristics of regenerable and non-regenerable maize tissue cultures, including those of **Duncan and Widholm (1989),** who showed that non regenerable type I callus, was darkly stained with K permanganate, while the regenerable callus was not. This was attributed to higher ferulic acid levels in regenerable type I callus (**Lozovaya *et al*. 1996**).

Metabolic profiling of regenerable and non-regenerable callus of both type I (H99) and type II (HiII) showed that the many biochemical differences between the morphotypes were generally similar with both H99 and HiII genotypes (**Lozovaya *et al*. 2006**). The non-permeable, nonmetabolized compound, polyethylene glycol (PEG), has been used for many studies with plants and cell cultures to simulate the effects of water stress (**Heyser and Nabors 1981; Ben-Hayyim 1987; Adkins et al. 1995; El-Tayeb and Hassanein 2000**). Generally, the effects seen are those expected for water stress, growth inhibition, induction of proline accumulation, and stress responses (**Rhodes *et al*. 1986; Fan *et al.,* 2006**).

Bohorova *et al.,* (1995) discussed the effect of the genotype on somatic embryogenesis. They detect the genetic background or genotype is main factor of the explants which was found to influence the regeneration potentiality.

These results also reports by **Tomes and Smith (1985)** and **Hodges *et al.* (1986)**, they indicated that the regeneration was genetically controlled by nuclear genes in maize.

Moreover, studies by **Willman *et al.,* (1989)** suggested that at least one gene or a block of genes controlled the expression of somatic embryogenesis of maize tissue cultures.

.Tissue culture will continue to play a key role in the genetic engineering process for the foreseeable future, especially in efficient gene transfer and transgenic plant recovery (**Hinchee *et al.* 1994**). The most common reason for post-zygotic failure of wide hybridization is embryo abortion due to poor endosperm development. Embryo culture has been successful in overcoming this major barrier as well as solving the problems of low seed set, seed dormancy, slow seed germination, inducing embryo growth in the absence of a symbiotic partner, and the production of monoploids of barley (**Raghavan 1980, 1994; Yeung *et al.* 1981; Collins and Grosser 1984; Zenkteler 1990**).

Immature embryos have been the most widely used explants for initiation of regenerable tissue cultures (**Armstrong and Green, 1985; Phillips *et al.*, 1988**). Immature embryos can initiate two types of callus cultures from their scutella surfaces: Type I and type II callus. Type I is compact and organogenic and easily obtained from immature embryos. On the other hand type II is friable and embryogenic and is initiated at a lower frequency than type I mature embryos of maize could be used to induce callus but no plantlets

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Tissue and cell culture systems are vital to many areas of plant science and crop improvement, particularly in mutant selection and plant transformation (**Phillips, 2004**). The ability to regenerate shoots from callus and cells is essential for establishing a successful plant culture system. However, because of restrictions in genotype and culture conditions, not all plant species or varieties can be regenerated easily (**Huang and Wei, 2004**).

Furini and Jewell (1994) suggested that callus obtained from immature embryos in presence of dicamba developed into somatic embryos than the callus obtained with 2,4-D. **Huang and Wei (2004)** mentioned the role of 2,4-D with MS media in inducing highly regenerable calli from mature embryos. Many published reports are available in maize suggesting successful regeneration from mature embryos **(Huang and Wei ,(2004),** split seeds **(Al-Abed *et al.* 2006)** other than use of immature embryos as explant **(Furini and Jewell 1994 and Bohorova *et al*.,1995).**

The history of maize tissue culture can be traced back to the early 1930's, when **Lampe and Mills (1933)** cultured young endosperm tissue as well as embryos on a medium supplemented with potato extract but observed only limited proliferation. The first continuously growing long-term tissue cultures of maize, initiated from immature endosperm, were obtained by **Larue (1947, 1949),** followed by more detailed studies on the physiology, morphology and cytology of endosperm cultures by others (**Straus and Larue, 1954; Straus, 1954, 1960; Tabata and Motoyoshi, 1965).**

Mature as well as immature embryos of maize were also cultured in order to determine their nutritional requirements for growth and development (**Larue, 1936, 1952; Haagen-Smit *et al*., 1945; Green *et al*., 1974**). None of these early studies were aimed at regenerating plants and none were recovered. Microprojectile bombardment system (MPS) proved to be a powerful technique for genetic modification of maize lines by delivering foreign DNA into scutellar tissues of immature embryos, reviewed by **O'Kennedy *et al*., (2001).**

Immature embryos have been extensively used for maize regeneration and transformation (**Green and Phillips, 1975**). This ability of immature embryos to produce embryogenic calluses makes them the most suitable primary explants for genetic transformation of maize (**Gordon-Kamm, 2002**). Nevertheless, the chance of using this kind of explants depends on the availability of embryos from plants growing in the greenhouse or the field. In certain working conditions this could be a critical point.

Zerihun *et al*. (2008) reported that use of immature zygotic embryos as an explant for maize regeneration has been hampered by the strictly limited suitable duration of immature embryos for culture. In contrast, mature zygotic embryos harvested from dry seeds are ubiquitous. The authors indicated that, however, generally mature embryos and especially tropical maize genotypes have been considered as the most recalcitrant for tissue culture work. Consequently tropical maize regeneration from mature embryos has not been reported so far. Here, they report successful regeneration of one inbred and one open pollinated tropical maize line from mature zygotic embryos using split seed technique. The results indicated that the maximum average callus induction recorded using LS basal salts and B5 vitamins supplemented with 3 mg l-1 2,4-D alone was 90% and 52.5% when same level of 2,4-D was combined with Kinetin. A maximum of 75.6% Type II and 62.3% Type I callus was produced after maintaining calli on media composed of LS basal salts and B5 vitamins supplemented with 2 mg l-1 of 2,4-D. also the showed the frequency of regenerable calli induced was 21.14% for CIMMYT maize line 216 and 16.51% for Katumani. The number of shoots regenerated per callus induced on single split seed.

Miroslava *et al*. (2011) reported that somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a somatic embryo. These characteristics have designated somatic embryogenesis into a model system for the study of morphological, physiological, molecular and biochemical events occurring during the onset and development of embryogenesis in higher plants. In their experiments somatic embryogenesis and plant regeneration was achieved from immature embryos of two maize (*Zea mays* L.) lines A18 and A19. The results showed that callus was initiated on N6 medium supplemented with 1 mg.dm-3 2,4-D, N6 salts, 2 % sucrose, 25 mmol.dm-3 proline, 100 mg.dm-3 casein hydrolysate, N6 vitamins, 10 mg.dm-3 silver nitrate, 3g gelrite. Also, results indicated that induction of primary callus ranged between 0 and 93%. Generally, three types of callus were formed: embryogenic, non-embryogenic and organogenic callus. The embryogenic callus was formed within two weeks of culture in callus maintenance medium. Induction of embryogenic callus ranged between 0 and 5%. Somatic embryos were matured on N6 medium supplemented with 6% sucrose and 1 mg.dm-3 NAA. After transfer of embryogenic calli on regeneration medium containing MS medium

supplemented with 2% sucrose, somatic embryos started to form plantlets. Callus initiation and plant regeneration were genotype dependent. Regenerated plants were transferred on the surface of solidified MS medium supplemented with myo-inositol.

Somatic embryogenesis is developmental process by which somatic cells undergo restructuring to generate embryogenic cells. These cells then go through a series of morphological and biochemical changes that result in the formation of somatic or non-zygotic embryo capable of regenerating plants. Somatic embryogenesis represents a unique developmental pathway that includes a number of characteristic events: dedifferentiation of cells, activation of cell division and reprogramming of their physiology, metabolism and gene expression patterns, reviewed by (**Zimmerman, 1993; and Komamine *et al*., 2005**).

Somatic embryogenesis forms the basis of cellular totipotency that is unique to higher plants. Differing from its zygotic counterpart, somatic embryos are easily traceable, culture conditions can by monitored and lack of material is not a limiting factor for experimentation (**Kawara and Komamine, 1995**). Somatic embryogenesis plays an important role in clonal propagation. When integrated with conventional breeding programs and molecular and cell biological techniques, somatic embryogenesis provides a valuable tool to enhance the genetic improvement of commercial crop species (**Stasolla and Yeung, 2003**).

Plant regeneration through tissue culture of maize was first reported by **Green and Philips (1975)** utilizing immature embryos as the explants. Since the successful plant regeneration has been reported from callus initiating from different tissue sources (**Ting *et al*., 1981; Rhodes *et al*., 1986;and Conger *et al*., 1987**). Maize immature embryos were most widely used as initial explant for maize regeneration (**Lu *et al*., 1982; Lu, Vasil, 1983; and Vasil *et al*., 1984**).

However various conditions for somatic embryo induction and regeneration were tested and used, the ability to regenerate embryo derived from callus cultures has been reported to be dependent on the maize genotype used (**Lee and Phillips, 1987; Obert *et al*. 2009**). Hormones are the most likely candidates in the regulation of developmental switches.

Auxins and cytokinins are the main growth regulators in plants involved in the regulation of cell division and differentiation. The influences of

exogenously applied auxins, preferentially 2,4-dichlorophenoxyacetic acid (2,4-D), on the induction of somatic embryogenesis are well documented (**Dudits *et al.*, 1995**). Auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the morphogenic processes. At the cellular level, auxins control basic processes such as cell division and cell elongation. Since they are capable of initiating cell division they are involved in the formation of somatic embryos.

These characteristics have made somatic embryogenesis a model for the study of morphological, physiological, molecular and biochemical events that occur during the onset and development of embryogenesis in higher plants. It also has potentially rich biotechnological applications such as artificial seed, micropropagation, transgenic plants, etc. (**Dudits *et al.*, 1995**).

A regeneration system was developed for elite Egyptian maize inbred lines using immature embryos as explants by **Hanaiya *et al.* (2003)**. This system proved to be highly genotype-dependent. Line Gz 643 was identified as the best line, revealing the highest regeneration frequency (42.2%). Addition of L-proline and silver nitrate to culture media greatly enhanced the formation of embryogenic type II callus and the regenerability of some of the tested lines. Transformation of the stellar tissue of immature embryos from inbred line Gz 643 was performed with the particle delivery system using a single plasmid carrying both the GUS and Bar genes (pAB-6) or by co-transformation with two plasmids, pAct1-F (GUS) and pTW-a (Bar). Different transformation parameters were evaluated, i.e. osmotic treatment, acceleration pressure, and number of shots.

Increasing interest in the production of biofuels has war ranted research in the production and genetic manipulation of high biomass crops such as switch grass (*Panicumvirgatum* L.), a warm season perennial grass native to North America. With this increased interest, it is necessary to develop higher throughput transformation systems that are enabled, by an efficient and reliable tissue culture, system for target tissue production and plant regeneration. Stable transformation, in turn, enables the reverse genetics research for cell wall manipulation and plant growth improvement.

Current switch grass tissue culture and transformation systems are not very efficient and limited to derivatives of a singl evariety: Alamo. There are currently two described tissue culture systems in switch grass: embryo genetic callus. **Denchev et al. (1994) and Dutta et al. (1999).**

      Moore (2008) reported that biofuels are criticized in the media for their low production energy efficiency, environmental impacts and by using food for fuel production. An answer most critics rely on is stating how generation biofuels will solve all the problems the first generation biofuels possess. However, generation biofuels must "pave the way" for generation biofuels. They can do this by providing the infrastructure, technology and knowledge provided by the fuels.

      Vasil and Vasil, (1986) summarized that, regeneration of plants from tissue cultures initiated from immature embryos and other explants of cereal species was first described. These included plant regeneration from cultured immature embryos and mesocotyl tissue of maize **by Green and Phillips (1975) and Harms *et al*., (1976).**

2.3. GENETIC ENGINEERING AND MOLECULAR GENETICS

Tissue-culture techniques are part of a large group of strategies and technologies, ranging through molecular genetics, recombinant DNA studies, genome characterization, gene-transfer techniques, aseptic growth of cells, tissues, organs, and in vitro regeneration of plants that are considered to be plant biotechnologies. The use of the term biotechnology has become widespread recently but, in its most restricted sense, it refers to the molecular techniques used to modify the genetic composition of a host plant, i.e. genetic engineering. In its broadest sense, biotechnology can be described as the use of living organisms or biological processes to produce substances or processes useful to mankind and, in this sense, it is far from new (**Zhong *et al.* 1995**).

The development of transgenic plants engineered for enhanced biofuel conversion is expected to be the most rapid and efficient solution, especially for the production of fuels from lignocellulosic biomass (**Gressel, 2008**). Two main techniques exist for producing transgenic plants, differing in the method by which the DNA of interest is inserted into the plant cell. The first transformation technique was through the use of the soil bacterium *Agrobacterium tumefaciens* to successfully introduce the bacterial gene encoding neomycin phosphotransferase type II (nptII) into plant cells. The second technology developed to transform plant cells utilized particle bombardment to physically shoot exogenous DNA into the plant cell (**Sanford, 2000**).

Any DNA can be coated onto tungsten microparticles in the 1 μm size range and bombarded into plant cells, where it appears to be integrated randomly into the plant genome by host factors. Biolistic transformation has successfully incorporated the chloramphenicol acetyltransferase (CAT) gene into maize cell cultures (**Klein *et a.l*, 1988**), and shortly after generated stable transgenic tobacco plants expressing the β-glucuronidase gene (**Klein *et al.*, 1988**). However, biolistic transformation often leads to integration of many (>10) copies or fragments of the transgene in the plant genome (**Wan Y, 1994**), which can sometimes lead to gene silencing (**Iyer, 2000**). With either using *Agrobacteria* or Biolistic transformation, the transformed plant cells must be regenerated into mature plants, a lengthy process that can take over six months depending on the plant species. Either transformation process can be used to generate the transgenic biofuel plants described below, though the choice of transformation technique can lead to different regulatory scrutiny.

The efficient and sustainable use of plant biomass for energy purposes remains a challenging endeavor, requiring major investments in science and technology. With the exception of sugar cane ethanol, biofuels are a nascent industry in many parts of the world. A few of the commercialized products include bioethanol derived from corn starch and biodiesel obtained from plants with a high content in fatty acids such as soybean, canola and sunflower. (**Schubert, 2007 and Himmel** *et al*. **2008**)

However, the status of corn and soybean as major food crops, coupled to the fact that yields of starch and plant oil are too modest to cover the huge demand of transportation fuels has prompted the development of alternative biofuel production based on lingo cellulosic biomas, (**Sticklen, 2008 and Tilman** *et al*. **2009).**

Genetic improvement of cereals has been a major focus of plant breeding efforts during the past 50 year, resulting in remarkable increases in the yield and improvement in the quality of this important group of food crops. Modern plant biotechnology had novel means for crop improvement through the integration and expression of defined foreign genes into plant cells, which can then be grown *in vitro* to regenerate whole plants. The efficient regeneration of normal and fertile plants from single cells, a basic prerequisite for the production of genetically transformed plants, proved to be rather difficult for gramineous species because of their extreme recalcitrance to manipulation *in vitro.* **Zhang** *et al.,* **(2002)**

The most likely near term transgenic plants that will be used for Biofuel production are those already on the market or those for which the traits were already under development for other uses. For example, many of the major seed companies have recently begun marketing high starch corn varieties for biofuel use.

Molecular genetics, genomic and biotechnological resources for candidate biofuel crops are limited at this time, but are growing in number. Many methods for reliable genetic transformation using *Agrobacterium* have been developed for switchgrass, *Jatropha*, poplar and *Brachypodium*, paving the way for genetic engineering approaches to crop improvement (**Xi and Wan, 2009**)

The successful engineering of a functional metabolic pathway for the production of polyhydroxybutyrate (PHB) in transgenic *switchgrass* has been

recently reported by **Somleva *et al.* (2009)**. They suggested that complex traits can be engineered in this dedicated biofuel crop.

Dyer and Fairless (2008) reported that plant seed storage oils, in the form of triacylglycerols (TAGs), are excellent sources for the generation of biodiesel due to their high chemical similarity to fossil oils and biodiesel is produced by the transesterification of plant TAGs with methanol in the presence of acid or alkali to produce fatty acid methyl esters (FAMEs). Current biofuel crops in addition, the quality of biodiesel produced is highly dependent on both the type and abundance of the fatty acids in seed storage organs. Thus, increasing oil content in plants and redirecting the biosynthesis of fatty acids for accumulation of specific types are needed to achieve optimal biodiesel production.

Pauly and Keegstra (2008) suggested that, plants deposit cellulose, hemicellulose and lignin in their cell walls. Recalcitrance is mainly due to the heterogeneity and molecular structure of lignocellulose, where cellulose is arranged into a network of tight, inter-chain hydrogen bonds that form a crystalline core of microfibrils, embedded in a matrix of hemicellulosic polysaccharides that are covalently linked to lignin, a highly complex polymer of aromatics. The study of how plant cell walls are synthesized, modified and degraded is one of the main areas of focus in biofuel research at the moment, with the aim of designing future bioenergy crops with improved lignocellulosic characteristics for easier and more efficient breakdown.

Plasticity can be exploited for engineering of novel lignin compositions for improved lignin extraction from biomass. For example, maize cell walls incorporating coniferyl ferulate had improved enzymatic hydrolysis and sugar release **(Vanholme *et al.*, 2008).**reported that modifying lignin content or structure can lead to severe developmental defects and to enhanced susceptibility to plant pathogens, while other plant cell wall modifications can actually increase resistance to biotic factors Importantly, although plant biotechnology will be key to the successful generation of energy crops, it should go hand in hand with breeding efforts targeted at maintaining or enhancing the important agronomic traits that made these plants so attractive for biofuel production to begin with, namely resistance to abiotic and biotic factors, low fertilization requirements and perennial life cycle.

Sticklen and Blanch *et al.* (2008) reported that the development of advanced biofuels from non-food crops is currently undergoing intensified

period of research, development, and commercialization because of the need to generate renewable and storable leliquid transportation fuels that can reduce green house gas emissions by displacing fossil fuels in addition, ,producing biofuels that are derived from lignocellulosic biomass (such as agricultural residues, timber, switch-grass, and Miscanthus) are expensive and difficult to generate efficiently due in large part to the inherent complexity and diversity of plant cell walls by (**simmon** *et al*.**, 2008 and sims** *et al*.**, 2010).**

Plant cell walls are composed of several components including cellulose, hemicellulose, lignin, pectin, structural proteins and aromatic compounds (**Carpita, 1996**). They are broadly classified as primary or secondary cell walls, which are distinguished by the absence or presence of lignin. Primary cell walls can be further classified into two types, namely Type I, and Type II. Type I cell walls are the most predominant found in dicot plants and in non-commelinoid monocots. Type II cell walls are found only in commelinoid monocots, which includes all of the cereal crops and perennial grasses (**Carpita and McCann, 2000**).

In Type I cell walls, cellulose microfibrils are interlocked by xyloglucan, the predominant hemicellulose. The cellulose-xyloglucan framework is then embedded in pectin and structural protein matrix. In Type II cell walls, the cellulose microfibrils are interlocked by a different hemicellulose, namely glucuronoarabinoxylan. An additional difference between these two types of cell walls is the presence of higher proportion of ferulic acid moieties on glucuronoarabinoxylan chains, which cross-link hemicellulose via diferulic acid diester bridges.

Secondary cell walls are characterized by the presence of lignin, which embeds the cellulose and hemicellulose matrix. Lignin in most of the dicots is composed of guaiacyl (G) and syringyl (S) units while in grasses lignin is composed primarily of p-hydroxyphenyl (H), guaiacyl and syringyl units (**Dixon** *et al*.**, 2001 and Grabber, 2005**).

Lignin is cross-linked by carbon-carbon (C-C) bonds or by ether linkages to form a complex network. Lignin also cross links cellulose and hemicellulose by ester and ether bridges and by diferulic acid diester-ether bridges in the case of grasses. The extensive cross-linking of cell walls by lignin is thought to be one of the key limitations for producing biofuels from biomass. Cell walls that are cross-linked are less accessible to hydrolytic enzymes, and necessitating physical or chemical pretreatment to allow hydrolysis to simple sugars.

In grasses such as maize, lignin is covalently linked to the hemicellulose glucurono-arabinoxylan via ferulate and diferulate bridges (**de Buanafi na, 2009; Hatfi eld** *et al.***, 1999; Ralph** *et al.***, 1995**). In turn, the hemicelluloses are non covalently associated with cellulose microfibrils via hydrogen bonds and van der Waals forces. These interactions result in a complex cell wall material that provides integrity and strength to both the plant cell and the plant itself.

One factor that influences the mechanical strength of the walls is the type of lignin subunits and their ratios within the lignin polymer. For example, a higher syringyl (S) content as found in hardwood cell walls increases the relative stiffness (**Koehler and Telewski, 2006; Reddy** *et al.***, 2005**).

The sheathing of the polysaccharides in the walls by lignin is considered a major impediment in the paper, pulp, and biofuels industries as it interferes with the enzyme accessibility necessary for the hydrolysis of cellulose and hemicelluloses (**Vermerris** *et al.***, 2010**).

Hence, a major hurdle in the economical and sustainable conversion of a lignocellulosic feedstock is the ease with which it is possible to chemically or physically open up the structure of the cell wall so that the hydrolytic enzymes are able to access the biomass polysaccharides (**Chundawat** *et al.,* **2010; Eggeman and Elander, 2005; Sticklen, 2008**).

Micro projectile bombardment system (MPS) proved to be a powerful technique for genetic modification of maize lines by delivering foreign DNA into scutellar tissues of immature embryos, reviewed by **O'Kennedy** *et al.***, (2001).**

Lerouxel *et al.* **(2006)** reviewed that lignocellulose, composed of the polysaccharides cellulose and hemicellulose, and lignin, a phenolic polymer, is the most abundant biomaterial on earth. Most lignocellulosic feed stocks in consideration are perennial, non-food grasses such as switch grass and *Miscanthus*, as well as woody plants such as poplar

More than 50 different plant species have already been genetically modified, either by vector-dependent (e.g. *Agrobacterium)* or vector-independent (e.g. biolistic, micro-injection and liposome) methods (**Sasson 1993 and Anon. 1994**).

In almost all cases, some type of tissue culture technology has been used to recover the modified cells or tissues. In fact, tissue-culture techniques have played a major role in the development of plant genetic engineering. For

example, four of the seven papers listed by **Davis and Reznikov (1992)** as classic milestones in plant biotechnology used a range of protoplast, microspore, tissue and organ culture protocols

Gordon-Kamm *et al. (***1990)** reported that biolistic gun (or particle gun) mediated DNA delivery was the first successful method used in producing fertile transgenic maize plants. "Biolistic" is a generic term for micro particle bom-bardment and derives from biological ballistic by **Armstrong, (1999).** In addition to **Klein, . (1987)** reviewed that, micro projectile bombardment is among the most reliable and efficient direct DNA delivery systems for monocots. The original design of the biolistic gun used a gunpowder charge to propel the DNA-coated tungsten particles (micro particles) through a vacuum chamber into target cells

Genetic breeding has been decisive and essential to transform maize into a major food crop worldwide. Biotechnology plays an important role in plant genetic breeding, particularly for the introduction of novel traits in order to improve agronomic performance, medical and industrial applications and food quality. In recent years, the development of efficient plant regeneration systems in cereal crops and the field of biotechnology have opened up new opportunities for genetic transformation of crop plants.

Some monocot plants were initially considered difficult for genetic engineering, primarily due to their recalcitrance to *in vitro* regeneration and their resistance to *Agrobacterium*. Continuous efforts and studies of different tissues for regeneration potential, development of various DNA delivery methods, and optimization of gene expression cassettes have led to the development of reliable transformation protocols for major cereals, including maize. Consequently, this research group has focused its attention on maize transformation mediated by micro projectile bombardment as a device of DNA delivery into maize cells. This method offers a rapid and simple way of introgression of candidate genes into cells. However, there are some points that still need to be studied and improved in order to achieve appropriate transformation efficiency to optimize the processing conditions to obtain fertile plants (**Cecilia** *et al***. 2010)**

About 20 years have elapsed since the initiation of the first experiments which led to the production of fertile transgenic maize plants. The commercial application of agricultural biotechnology in corn has primarily focused on the development of input traits that can provide attributes beneficial to the grower.

However, in order to obtain high-quality and high-frequency transformation systems, transformation techniques need to be optimized frequently. Particle bombardment offers a rapid method for DNA delivery into plant cells (**Rasco-Gaunt *et al*., 1999**).

Particle acceleration can be achieved through High Pressure Gene Gun (HPGG) or Low (LPGG) helium pressure gene guns (**Li *et al*., (2003).** Several factors affect the transformation efficiency of gene guns (**Décima *et al*., 2010**), and establishing changes in the transformation protocols is critical in order to obtain an adequate technique that fits with the lab resources and difficulties.

The plant transformation technique using the helium pressure gun involves inert particles such as gold or tungsten coated with DNA. The particles go through the plant cell membranes to reach the nucleus and then integrated into the plant genomic DNA. The transformation efficiency depends on several variables: the explant genotype, the helium pressure, the particle size, the *in vitro* culture capacity and explants regeneration, the plant adaptation to *ex vitro* conditions and the seed production capacity (**Zhang *et al*., 2007**).

Particle bombardment and *Agrobacterium* mediated transformation are two popular methods currently used for producing transgenic cereals the application of *Agrobacterium* mediated transformation to monocotyledonous species, including rice and maize, has been recently reported (**Shouh *et al*. 2004**). The main characteristics of the *Agrobacterium* system in these species are the high frequency of transformation; proper integration of the foreign gene into the host genome and low copy number of the gene inserted, resulting in most cases in a correct expression of the transgene itself.

There has been a rapid increase within the last twenty years in the number of researchers involved in the aseptic culture of plant cells, tissues, and organs; that is, "plant tissue culture". The reasons are twofold: (1) important technical developments have allowed for greater reproducibility of experimental results, and (2) numerous investigations have demonstrated the utility of these techniques in the study of many biological questions especially in genetics, physiology, and development (**Chaleff and Carlson 1974; Day 1977; Murashige 1978; Smith 1974; Sprague *et al*. 1980 and Zenk 1978**).

Cellulosic ethanol is a desirable alternative to corn because it has a higher potential yield. While sugar is fermented in both types of ethanol production, cellulose has a higher glucose content, which makes it easier to form sugar (grain-based ethanol must first be converted from starch to sugar). Cellulose

additionally contains both five- and six-carbon sugars, called pentoses and hexoses, which can be utilized by various microorganisms. **(Ethanol Across America, 2009)**

Biomass is typically composed of about 40% to 50% cellulose, 25% to 35% hemicellulose, and 15% to 20% lignin, which are substances that are not broken down by the enzymes used in corn ethanol production (Peterson and Ingra 2008). Cellulosic ethanol can be produced from various energy crops **Service, 2007), Peterson and Ingra, 2008), Schmer *et al*., 2008)** studies that.

Hanaiya *et al.,* (2003) developed a regeneration system for elite Egyptian maize inbred lines using immature embryos as explants. They reported that this system proved to be highly genotype-dependent. Their results indicated that line Gz 643 was identified as the best line, revealing the highest regeneration frequency (42.2%). Addition of L-proline and silver nitrate to culture media greatly enhanced the formation of embryogenic type II callus and the regenerability of some of the tested lines. Transform ation of the scutellar tissue of immature embryos from inbred line Gz 643 was performed with the particle delivery system using a single plasmid carrying both the GUS and Bar genes (pAB-6) or by co-transformation with two plasmids, pAct1-F (GUS) and pTW-a (Bar). They evaluated different transformation parameters, i.e. osmotic treatment, acceleration pressure, and number of shots. Their results clearly showed that osmotic treatment of 0.25M sorbitol and 0.25M of mannitol along with the use of either acceleration pressure 1300 psi and one shot per plate (for co-transformation with pAB-6) or 1100 psi and two shots per plate (for transformation with pAct1-F and pTW-a) gave the best results, as expressed by the number of blue spots in the b-glucuronidase (GUS) assay.

In the 1980s*, Agrobacterium*-mediated gene transfer emerged as the most straightforward way to generate transgenic dicotyledonous plants, but few monocots were amenable to this process. Efforts to transform maize therefore began with physical delivery systems targeting protoplasts **(Fromm *et al*., 1985; Klein *et al*., 1987).** The first transgenic maize plants produced by protoplast transformation were sterile **(Rhodes *et al*., 1988)** but fertile transgenic plants were eventually generated by bombarding suspension cell cultures from inbred line A188 with DNA-coated metal particles **(Gordon-Kamm *et al*., 1990).**

In 1996, fertile transgenic plants from the same inbred line were produced by *Agrobacterium-mediated* transformation using a strain carrying a "super

binary" vector system (**Ishida *et al*., 1996**). Additional methods that have been used to generate transgenic maizeplants include PEG-mediated protoplast transformation (**Golovkin *et al*., 1993**), electroporation of immature embryos or embryonic callus cultures (**D'Halluin *et al*., 1992**) and the transformation of embryogenic cell suspensions (**Frame *et al*., 1994**) or callus cultures (**Petolino *et al*., 2000**) using silicon carbide whiskers-mediated transformation and particle bombardment remain the most widely used methods for transformation.

Biomass to be metabolized into ethanol, it must first be broken down into its sugar-based components. One method of doing so uses enzymes. Breaking down the feedstock is what makes cellulosic ethanol more complicated to produce compared to corn. Corn is comprised of starch, a polymer of glucose that is easily broken down by enzymes called amylases. Biomass, on the other hand, is composed of three different molecules: cellulose, the main component of plant cell walls, which is a complex polymer of glucose that contains strong hydrogen bonds; hemicellulose, a branched, amorphous polymer of pentoses and glucose; and lignin, a non-sugar molecule that encapsulates other polymers and provides robust structure **Demirbas, 2005, Service, (2007), Sticklen, Woodson, and *et al*. (2008).**

Sanford (2000) reported that particle bombardment is a commonly used method for genetic transformation of plants and other organisms. Millions of DNA-coated metal particles are shot at target cells or tissues using a biolistic device or gene gun. The DNA elutes off the particles that lodge inside the cells, and a portion may be stably incorporated in the host chromosomes. Particle bombardment employs high-velocity micro projectiles to deliver substances into cells and tissues. For genetic transformation, DNA is coated onto the surface of micron-sized tungsten or gold particles by precipitation with calcium chloride and spermidine. Once inside the cells, the DNA elutes off the particles. If the foreign DNA reaches the nucleus, then transient expression will likely result and the transgene may be stably incorporated into host chromosomes

Although the protein content of maize is relatively high (9% on average), its quality is poor due to an imbalance in three essential amino acids, in which the contents of tryptophan and lysine are low, whereas that of leucine is high. This amino acid balance ratio is not enough to satisfy the FAO requirements for human nutrition, especially for children (Hamaker and Rahmanifar 1994).

DNA transfer via particle bombardment is currently the most widely used method for wheat transformation, and the stable expression of transgenes has

now been achieved by several groups (**Vasil *et al.* 1992; Weeks *et al.* 1993; Becker *et al.* 1994; Nehra *et al.* 1994; Zhou *et al.* 1995; Altpeter *et al.* 1996; Ortiz *et al.* 1996; Barro *et al.* 1997**). However, widespread application of the technology is still limited by relatively low and erratic stable transformation efficiencies and by the general use of tissue culture-responsive but agronomically less desirable "model" genotypes such as 'Bobwhite' and 'Florida'.

One of the major problems with all biolistic devices developed so far is the use of high pressure, which causes severe damage to target cells. They also suffer from several other disadvantages: (1) low transformation efficiency, (2) need to use large amounts of deoxyribonucleic acid (DNA), (3)laborious to use, (4) high noise level, (5) large size and design makes it less portable, (6) and the high cost of the basic instrument and disposable supplies needed for operation. The low-pressure Bio Ware gene gun has been used for genetic transformation of animal cells and human tumor cell lines only (**Chang *et al.* 2008; Cheng *et al.* 2005a, b; Tu *et al.* 2007).**

Genetic improvement of cereals has been a major focus of plant breeding efforts during the past 50 year, resulting in remarkable increases in the yield and improvement in the quality of this important group of food crops. modern plant biotechnology had novel means for crop improvement through the integration and expression of defined foreign genes into plant cells, which can then be grown *in vitro* to regenerate whole plants. The efficient regeneration of normal and fertile plants from single cells, a basic prerequisite for the production of genetically transformed plants, proved to be rather difficult for gramineous species because of their extreme recalcitrance to manipulation *in vitro.* **Zhang *et al.* (2002)**

The presence of lignin is one of the most significant contributors to biomass recalcitrance and consequently increases the costs associated with conversion .In addition, there are two basic approaches being examined to reduce cell wall recalcitrance, largely because of lignin to enzymatic saccharification (**Kim and Ralph *et al.* 2010).** The first is logically to perturb the known lignin biosynthetic pathway, to alter the lignin content, composition, and structure to decrease, often empirically, the severity of required pre-treatment conditions. The second is an attempt to fun-damentally redesign lignin, in a hopefully plant-compatible manner, specifically for easier processing and at the same time help to convert lignin into more valuable

aromatic chemicals. Both approaches are leading to an enhanced understanding of lignin biosynthesis, and its interaction with other plant processes, as well asimproving our knowledge of, and the options available for, plant cell wall deconstruction.

Escamilla and Jansson *et al.* (2010) used lignin to generate feeds tocks with diminished recalcitrance, researchers have employed several strategies using different plant species are model systems for lignocellulosic biofuel crops while switchgrass, rice and maize

Vogt (2010) reviewed that the development of genomic tools for other biofuel crop species will provide new avenues in terms of manipulating lignin for improved biofuel con-version. Genetic engineering of several food crop. maize species is already a mature practice via the gene-gun-mediated or by *Agrobaterium tumefaciens*-mediated transformation approaches.

Armstrong *et al.* (1991) discussed that transformation amenable group of lines by crossing to cytoplasmic male sterile (CMS)-T (Texas) maize. The first report of successful transformation of such male sterile lines using the Biolistic (gene gun) or *Agrobacterium* mediated methods. The second strategy involves transfer of the subunit vaccine gene from a fertile transgenic line to acms-TB37 background. After six seasons of breeding, male sterile transgenic maize seed can be used for open-field production using a non-transgenic pollen donor to produce seeds for large scale re-com bin ant protein recovery, 100% of which contain the subunit vaccine gene.

Pimentel (2009) indicated that, the use of corn to produce ethanol is not entirely to blame. At a global scale, only about 4% of the world's grain is used to make biofuels, but this detraction from the food market coupled with market forces and government subsidies is believed to contribute to huge increases in the price of food

.**Hertel *et al.* (2010) s**ummarized that corn-based ethanol makes up 97% to 99% of all biofuels in the US, the supply of corn is limited, and it also detracts from food corn sources, hitting those that are already malnourished the hardest.

Wang (1987) successfully regenerated plants from mature embryos of two maize inbreeds, B73 and Mo17, but the regeneration was genotype dependent and the frequency was only 4 to 5%. **Huang and Wei (2004)**

reported regeneration of temperate maize lines from mature embryos at a frequency ranging from 19.85 to 32.4%.

Most recently **Al-Abed et al. (2006)** reported more efficient regeneration system for two hybrid and two inbred temperate maize lines using split mature seeds as an explant. Identification of genotypes that respond well to embryogenic callus induction and plant regeneration is a necessary initial step for their successful genetic transformation.

Sticklen, (2008) reported that, cellulose is a polysaccharide that is found in the cell walls of plants. Globally, 180 billion tons of cellulose are produced per year, making it the largest organic carbon reservoir on the planet

Maize plant regeneration can take place through two avenues, that is, organogenesis or somatic embryogenesis. Somatic embryogenesis is the most common avenue of plant regeneration **(Odour et al. 2006).** With the rapid development of tissue culture techniques, many types of explants, including gametic embryo and leaf tissue had been successfully regenerated into plants by tissue culture **(Aulinger et al. 2003, Huang and Wei 2004 and Ahamadabadi et al. 2007).** But at present, the most popular is still immature zyotic embryo in maize transformation, owing to simple inoculation operation and facile callus induction **(Binott et al. 2008).**

Thus, screening of genotypes for in vitro plant regeneration is always a very important research task. Regarding the optimization of media composition, there have been many elite media compositions in literatures **(Du et al., 2007; Binott et al., 2008; Zhang et al., 2008),** but to be mentioned, any media containing specific reagents was only suitable for limited materials. Thus, to develop a new media for given materials is always necessary and significant

Philippe et al. (1993) summarized the effects of osmotic conditioning on both transient expression and stable transformation by introducing plasmid DNAs via particle bombardment into embryogenic suspension culture cells of *Zea mays* (A188 x B73). Placement of cells on an osmoticum- containing medium (0.2 M sorbitol and 0.2 M mannitol) 4 h prior to and 16 h after bombardment resulted in a statistically significant 2.7-fold increase in transient B-glucuronidase expression. Under these conditions, an average of approximately 9,000 blue foci were obtained from 100 p.l packed cell volume of bombarded embryogenic tissue. Osmotic conditioning of the target cells resulted in a 6.8-fold increase in recovery of stably transformed maize clones. Transformed fertile plants and progeny were obtained from several transformed

cell lines. The authors indicted that the basis of osmotic enhancement of transient expression and stable transformation resulted from plasmolysis of the cells which may have reduced cell damage by preventing extrusion of the protoplasm from bombarded cells.

Particle bombardment is valuable for both gene expression and stable transformation research (**Christou *et al.* 1988**). The basis of particle bombardment is the acceleration of small DNA-coated particles toward cells resulting in the penetration of the protoplasm by the particles and subsequent expression of the introduced DNA. With certain plants, particle bombardment is currently the most efficient method for introduction of foreign DNA. Although there have been many reports on optimization of physical bombardment parameters (**Klein *et al.* 1988**) and modification to the actual bombardment device (**Williams *et al.* 1991; Sautter *et al.* 1991; Finer *et al.* 1992**), limited data has been reported on cell preparation methods to make the target tissue more receptive to particle gun-mediated transformation. Benefits from culture venting (**Russell *et al.* 1992**), cell filtration (**Finer *et al.* 1992**), and the use of cells in the proper phase of growth (**Armaleo *et al.* 1990**) or at the proper density (**Finer *et al.* 1992**) have been reported for different species using the particle gun

Particle gun-mediated transformation of *Zea mays* has been reported by several laboratories (**Fromm *et al.* 1990; Gordon-Kamm *et at.* 1990; Waiters *et al.* 1992**) and is currently the most efficient technique for production of fertile, transgenic maize plants. The authors described the effect of osmotic treatment on transient expression and stable transformation of embryogenic maize cells and the recovery of fertile transgenic maize plants

2.4. LIGNIN MODIFICATION

Lignin itself is a complex polymer of phenylpropanoid units that hardens the cell walls of xylem tissue to provide mechanical strength to the stems and provides a physical barrier against pests and pathogens. Because lignin is hydrophobic, it decreases the permeability of cell walls and facilitates the transport of water through the xylem tissue reporters by **Higuchi, T, (1985).**

Li   et al. (2008) reported that**,** Lignin is a major component of plant cell walls and impedes enzymatic hydrolysis of the cellulose and hemicellulose to fermentable sugars. There is an inverse relationship between lignin content/composition and plant cell wall enzymatic   hydrolysisand fermentation kinetics.

Saathoff *et al.*, (2011) reviewed that**,** down-regulation of cinnamyl alcohol dehydrogenase (CAD), the last enzyme of lignin precursor formation, increases scarification efficiency up to 23% without acid pretreatment. In addition to **Fu *et al..,* (2011)** reported that, Likewise, down-regulation of caffeic acid 3-O-methyltransferase (COMT), a key en-zyme for biosynthesis of the monolignol sinapyl alco-hol, increases saccharification efficiency by 29-38%without acid pretreatment.

Klinke *et al*., (2004) reviewed that the reduction of sinapyl monolignol production may increase concentrations of fermentation inhibitors**.** While**, Tschaplinski *et al* (2012)** showed that, low molecular weight phenolic compounds in COMT down-regulated switchgrass inhibit simultaneous saccharification and fermentation (SSF) by the yeast Saccharomyces cerevisiaeun-less first removed by hot water pretreatment

Hisano *et al*. (2009) reported that**,** since the industrial revolution, mankind has exploited fossil energy sources for manufacturing and transport. Depletion of petroleum reserves, geopolitical tension, and climate change have increased the need for alternative and sustainable sources of energy.

Yuan *et al*., (2008) detected that the potential alternatives, besides solar radiation and wind, is lingo cellulosic biomass of which the sugar fraction in the secondary cell wall (cellulose and hemicelluloses)can be used for the production of liquid biofuels, such as bioethanol.

Chen *and* DIXON (2007) reviewed that the enzymatic processing of plant biomass into fermentable sugars, called saccharification, is hampered by the complexity of the secondary cell wall structure and the presence of lignin

Endler *et al*., (2011) summarized that the major component of the secondary cell wall is cellulose are a polymer of 1,4-linkedβ-D-glucose units, of which the largest fraction is organized into micro fibrils through inter- and intramolecular hydrogen bonds and van der Waals forces. The fraction of the micro fibril-bound cellulose is called crystalline cellulose to distinguish it from the remaining 'unorganized' cellulose, called amorphous cellulose

Gírio *et al*. (2010) studied the spaces between individual cellulose micro fibrils are largely filled with hemicelluloses that are far more complex in sugar com-position and linkage types than cellulose. In angiosperms, the major hemicelluloses are glucuronoxylans, xyloglucans, and glucomannans.

Scheller and Ulvskov (2010) reported that Glucuronoxy lansconsist of a linear backbone of 1,4-linked β-D-xylopyranosyl units that are substituted with acetyl and4-O-methylglucuronic acid side chains. Xyloglucans aremade of a D-glucose backbone of which 75% of the resi-dues are substituted by D-xylose. In addition, L-arabin-ose and D-galactose residues can be attached to thexylose residues.

Boerjan *et al*. (2003) and Vanholme and Demedts, (2010) reported that The third major type of polymer in the secondary cell wall is lignin. In dicotyledonous plants, lignin is mainly made from the mono lignols coniferyl alcohol and sinapyl al-cohol and traces of p-coumaryl alcohol that give rise to guaiacyl (G), syringyl (S), and p-hydroxy phenyl (H) units. Most of these units are linked via ether bonds (in so-calledβ–O–4-structures) and carbon-carbon bonds [in resinol(β–β), and phenylcoumaran (β–5) structures.

Himmel *et al*. (2007) reported that, themono lignols are synthesized from phenylalanine throughthe general phenylpropanoid and mono lignol-specific path-ways (Figure 1). After the monolignols are transported to the cell wall, they are oxidized by laccases and peroxidasesto monolignol radicals that then couple in a combinatorialfashion, finally generating the lignin polymer.To tailor biomass for improved cell wall deconstruc-tion, a profound knowledge is required of the factorsthat determine the recalcitrance of cell walls to sacchari-fication.

Chen *et al*. (2012) detected the major factors causing recalci-trance to saccharification is the presence of lignin, as shown in a series of transgenic alfalfa (*Medicago sativa*) lines with variable lignin amount and composition and, later on, in tobacco (*Nicotiana tabacum*) maize (*Zea mays*)
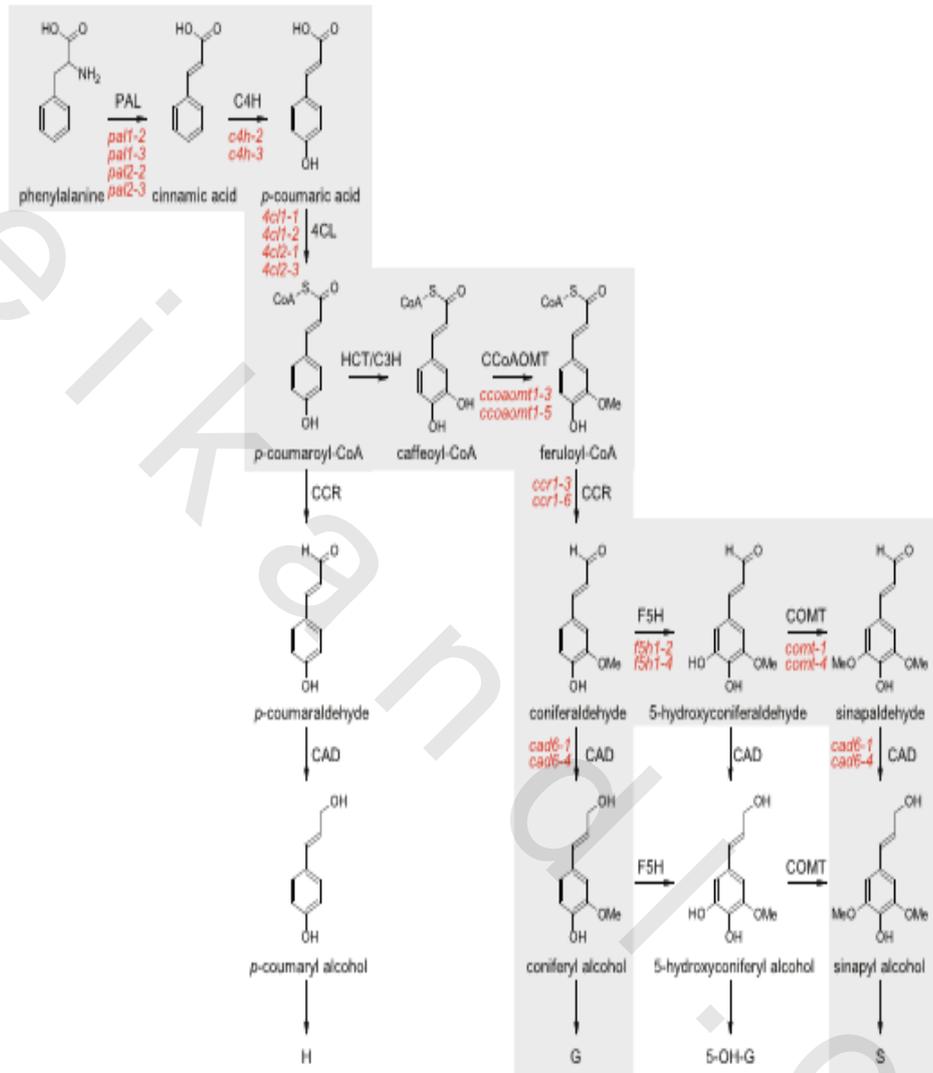

Figure (1) Phenylpropanoid and monolignothetic pathways. The grey box represents the phenylpropanoid and monolignol biosynthetic pathways that are generally accepted for angiosperms with indication of the 20 mutant alleles studied (in red). The general phenylpropanoid pathway starts with PAL and ends with CCOAOMT, whereas the monolignol-specific biosynthesis starts with CCR

Jouanin *et al* (2000) suggested that Cell wall engineering is complicated by our limited knowledge about potential crosstalk between the biosynthetic pathways of the main cell wall polymers. For example, several reports have suggested that a reduced lignin amount is compensated for by an increase in cellulose, as observed in poplars down-regulated in 4-coumarate: CoA ligase (4CL) and caffeic acid O-methyl transferase (COMT).

Vanholme *et al*. (2012) analyzed the amount and composition of composition of cell wall polysaccharides, analyzed the amount and composition of the three main cell wall polymers (lignin, cellulose, and matrix polysaccharides)of the senesced inflorescence stems of Arabidopsis mu- tants, mutated in 10 different genes of the phenyl pro-panoid and mono lignol biosynthetic pathways, i.e.,two mutant alleles of each gene encoding phenyl lanineammonia lyase 1 (PAL1), PAL2, cinnamate 4-hydroxylase(C4H), 4CL1, 4CL2, caffeoyl-CoA O-methyltransferase1(CCOAOMT1), cinnamoyl-CoA reductase 1 (CCR1),ferulate 5-hydroxylase 1 (F5H1), COMT, and cinnamyl al-cohol dehydrogenase 6 (CAD6). In addition, developed a small-scale saccharification protocol for Arabidopsisin-florescence stems and analyzed whether the altered cell wall composition in the mutants affected the saccharifica-tion yield. Analysis by whole cell wall Nuclear Magnetic Resonance (NMR) methods provided comparable findingson the lignin composition. The high number of mutants and biological repli-cates allowed us to draw correlations among cell wall composition

Yoshida *et al*. (2008) and Studer *et al*. (2011) evaluated the Miscanthus sinensisand Populussp with varying lignin content and/or alteration of lignin composition showed that sugar release increased as lignin content decreased.

Chen and (2007) reported that the evaluation of transgenic lines of al-falfa down-regulated in the lignin pathway has shown increased sugar release from hydrolysis in comparison to the wild-type, and this phenomenon is directly related to the reduction of lignin content. Moreover, the reduction of ferulate-lignin cross-linking or lignin content improved ruminal fermentation performance **(Grabber and Mertens, 2009)**

Fu *et al*. (2011) detected that, a transgenic switchgrass (*Panicum virgatum*) with down-regulation of the COMT (caffeic acid 3-O-methyltransferase) gene showed improved susceptibility to bioconversion using yeast-based simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) with C. Thermocellum.

Humphreys and Chapple (2002) reviewed that lignin biosynthesis in which p-coumaric acid, ferulic acid and sinapic acid acts as precursor to the formation of p-hydroxyphenyl, guaiacyl and syringyl lignin, respectively was challenged in the last decade. Identification of caffeoyl CoA 3-O-methyltransferase (CCoAOMT) as a key enzyme that converts caffeoyl CoA to feruloyl CoA, and its role in lignin biosynthesis suggested an alternative to free acid pathway **By (Ye *et al.,* 1994; Zhong *et al.,* 1998).**

Many authors such as **Humphrey *et al.,* (1999, Osakabe *et al.,* (1999), Li *et al.,* (2000), Parvathi *et al.,* (2001)** detected that, Subsequent characterization of ferulate 5 hydroxylase (F5H) and caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) enzyme activity in Arabidopsis and other species suggested that F5H and COMT displayed greater affinity for coniferaldehyde and 5-hydroxyconiferaldehyde, respectively than ferulic and 5-hydroxyferulic acid as originally hypothesized**.**

Schoch *et al.* (2001) explained a key finding in our understanding of lignin biosynthesis is the characterization of the p-coumarate 3-hydroxylase activity that was believed to occur at the level of free acids through the conversion of p-coumaric acid to caffeic acid. CYP98A3, a candidate gene for C3H activity, converted p-coumaroyl shikimate and p-coumaroyl quinate into their corresponding caffeic acid conjugates and did not prefer p-coumaric as a substrate. analyzed the amount and composition of characterization of Arabidopsis, alfalfa and poplar CYP98A3 by mutant or transgenic approaches showed that CYP98A3 is involved in lignification

(Franke *et al.,* 2002; Nair *et al.* 2002; Reddy *et al.,* 2005, Coleman *et al.,* 2008).

A gene that encodes for hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) activity, which converts p-coumaryl CoA to p-coumaroyl shikimate or p-coumaroyl quinate was also identified and its role in lignin biosynthesis was unequivocally established**. (Hoffmann *et al.,* 2003 and 2004).**

Nair *et al.* (2004) suggested that the free acid pathway in which caffeic and sinapic acid act as precursors to lignin biosynthesis was incorrect. Additionally, Arabidopsis ref1 mutant characterization further suggested that ferulic acid and sinapic acid are partly derived from coniferaldehyde and sinapaldehyde respectively by the action of an aldehyde dehydrogenase.

Halpin *et al*. **(1998)** detected that the precursors of lignin are synthesized via the phenylpro-panoid pathway. The first step in the pathway is the deamination of phenylalanine to produce cinnamic acid. Through a number of hydroxylation and methylation reactions and a reduction of the carboxyl moiety, cinnamic acid is converted to p-hydroxy-cinnamyl alcohols, also referred to as monolignols, which can then undergo polymerization within the cell wall. Detailed analysis of the cell wall composition of the bmmutants can provide information on the role of specific genesin lignin biosynthesis and can improve our understanding of lignification. The bm1mutation was shown to affect the activity of the enzyme cinnamyl alcohol dehydrogenase (CAD).

Pyrolysis-mass spectrometry studies indicated that the lignin in abmumutant showed a lower degree of cross-linking and that thevascular tissue in the mutant was lacking the tissue-specific cellwall composition observed in the wild-type plants (**Vermerris,** *et al.,* **(2001).**
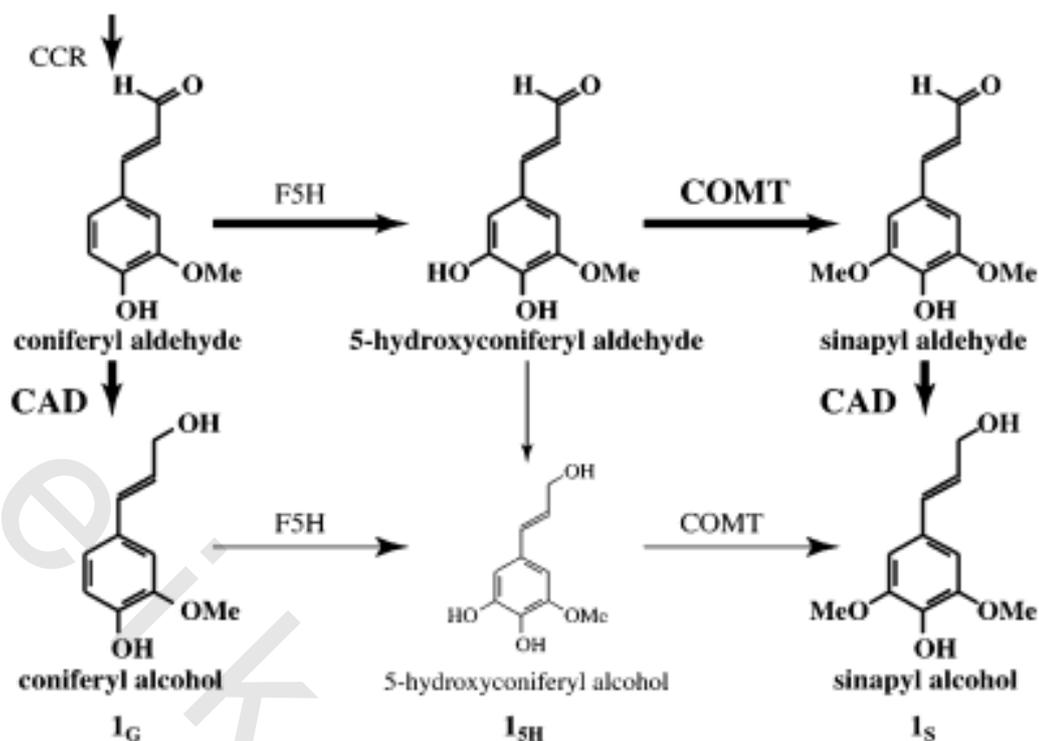
Figure (2) Partial diagram of the monolignol pathway in angiosperms. Bolder structures and large-diameter arrows represent preferred pathways as recently clarified (53−55). Thebm1mutation affects the cinnamyl alcohol dehydrogenase (CAD) enzyme activity, and the bm3 mutation affects the caffeic O-methyltransferase (COMT) enzyme activity