Chapter Number

Innovative Biological Solutions to Challenges in Sustainable Biofuels Production

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1. Introduction

The rising prices, declining supplies, and concerns about environmental safety and energy security associated with the use of fossil fuels are driving the development and use of biofuels (Gonzalez-Garcia *et al.*, 2010; Markevicius *et al.*, 2010; Singh *et al.*, 2010; Sahin, 2011). Biofuels in general can be defined as liquid, gas and solid fuels predominantly produced from biomass (Demirbas, 2008). In this chapter, we will specifically focus on liquid biofuels which have attracted world-wide attention due to their renewability, sustainability, common availability, reduction of greenhouse gas (GHG) emissions, and biodegradability (Demirbas, 2009; Gonzalez-Garcia *et al.*, 2010; Balat, 2011). Currently there are two major types of liquid biofuels, bioalcohol and biodiesel, as alternatives to gasoline and diesel fuel, respectively. Among the various bioalcohols, bioethanol is currently the most widely used and biobutanol has great growth potential in the future due to its significant properties including high energy content, hydrophobicity, blending ability, compatibility with combustion engines, corrosion, and octane rating (Kumar & Gayen, 2011). To date, liquid biofuels have been mainly produced in the U.S., Brazil and several European countries (Fig. 1A). Furthermore, there is a regional difference in the preference for biofuels types, with bioethanol preferentially produced in the American and Asian countries (e.g., U.S., Brazil, China, and Canada) while biodiesel is preferentially produced in European countries (e.g., Germany, France) (Fig. 1B).

Bioethanol can be produced from three categories of raw materials: simple sugars, starch, and lignocelluloses (Balat, 2011). Biomass feedstock for biodiesel production is under active development worldwide, with rapeseed and sunflower oils predominating in Europe, palm oil in tropical countries, and soybean oil and animal fats in the United States; and development of additional feedstocks such as *Jatropha* oil and algae for biodiesel is also underway (Dyer *et al.*, 2008; Knothe *et al.*, 2009). In particular, microalgal oil is one of the major renewable biofuels with great potential for replacing petroleum-based liquid fuels (Cooper *et al.*, 2010).

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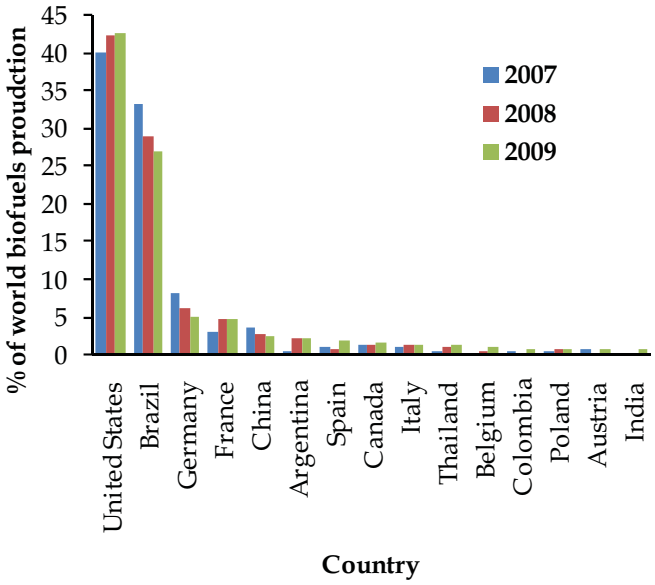
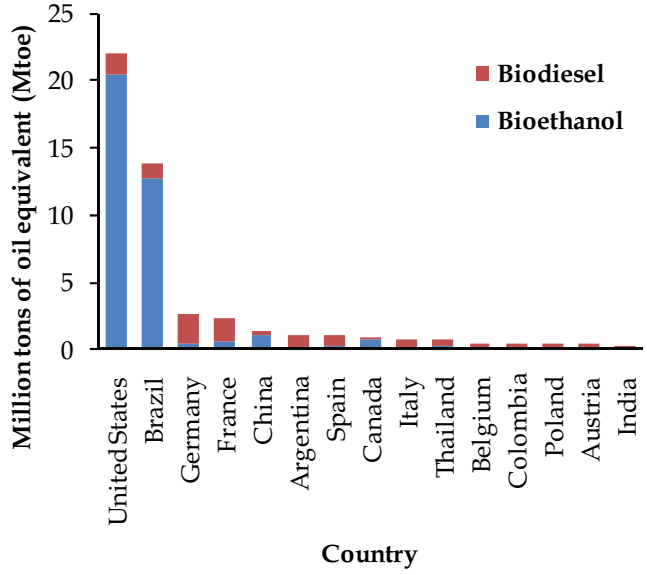


(a)



(b)

Fig. 1. World-wide production of biofuels. (A) Distribution of production of liquid biofuels (i.e., bioethanol and biodiesel) in the years 2007 – 2009, and (B) production of bioethanol and biodiesel in the year 2009. Drawn from data obtained from http://www.plateforme-biocarburants.ch

Although biofuels have advantages over fossil fuels, the use of biomass does not automatically imply that its production, conversion, and utilization are sustainable given the potential conflict between land use for food versus fuel (Markevicius *et al.*, 2010; Payne, 2010). In this chapter, we will first describe the challenges in the sustainable production of liquid biofuels and then discuss the novel biological approaches for solving these challenges.

2. Challenges in sustainable biofuel production

Currently sustainable biofuel production faces several major challenges: 1) Biofuel versus food competition, 2) limited biomass production, 3) recalcitrance of biomass for biofuel production, and 4) less-than-ideal physical properties of biofuels. We will discuss each of these challenges below.

2.1 Biofuel versus food competition

Biofuel crops are generally planted on agricultural land and most of the current bioenergy crops are also used as food or animal feed. Such dual-use crops include barley, maize, rice, rye, sorghum, wheat, cassava, potato, sugar beet, sugarcane, rapeseed, and soybean (Gerbens-Leenes *et al.*, 2009; Sahin, 2011). To date, almost all bioethanol has been produced from food sources such as grain or sugarcane (Mussatto *et al.*, 2010; Somerville *et al.*, 2010) and expanding biofuel production from such feedstocks is likely to exacerbate food insecurity and political instability (Payne, 2010). If terrestrial biofuels are to replace ~90 EJ (= 90×10^{18} J) mineral oil-derived transport fuels, large areas of good agricultural land will be required: about 5×10^{8} ha in the case of biofuels from sugarcane or oil palm and at least 1.8-3.6×10^{9} ha in the case of ethanol from wheat, corn, or sugar beet, an area that is equivalent to the current worldwide cropland (~1.8×10^{9} ha) (Reijnders, 2009). Moreover, bioenergy crops will potentially compete with food crops for inputs such as water and nutrients. Agriculture accounts for ~70% of all the world's freshwater withdrawals (Rosegrant *et al.*, 2009) and a decline in water availability is already a major constraint on agricultural productivity and global food security (de Fraiture *et al.*, 2008). Thus, sustainable production of biofuel feedstocks requires the use of land that is not required or is not suitable for food production (Marko *et al.*, 2009; Reijnders, 2009; Fritsche *et al.*, 2010). Development of new capabilities for biomass production on marginal or abandoned land with minimized water and nitrogen supply would be the best strategy to avoid the biofuel versus food competition. We will discuss several specific approaches to implement this strategy, such as introducing of new crops (see Section 3.1) and transgenic crops (see Section 3.2) that have high water use efficiency (WUE) and nitrogen use efficiency (NUE).

2.2 Limited supply of biomass for biofuel production

A major constraint on bioethanol production is the availability of biomass feedstock (Balat, 2011). Currently biofuel production accounts only for a small portion (~2%) of the 1,200 billion liters of annual gasoline consumption worldwide (de Fraiture *et al.*, 2008) and the contribution of biodiesel to global transportation fuel consumption is only 0.14% (Courchesne *et al.*, 2009). Assuming that 50% of the energy content of the feedstock can be recovered as liquid biofuels, the potential of global woody biomass is predicted to produce 73.8 million tonnes (3.1 EJ) of liquid biofuels in the year 2020, accounting for only 2.6% of the

global forecasted transportation fuel consumption (117 EJ) (Asikainen, 2010). The production of biofuels from lignocellulose is limited by the amount of plant biomass, as demonstrated by the estimation that lignocellulosic biomass harvested from all switchgrass, hybrid poplar, corn stover, and wheat straw in the United States could produce 10.31 billion gallons of ethanol or 8.27 billion gallons of butanol, which could replace 6.97 or 7.55 billion gallons of gasoline, respectively, leaving a significant gap from the target of 21 billion gallons of biofuels per year (Swana *et al.*, 2011). The major economic factor affecting the input costs of biodiesel production is the feedstock, which is about 75-80% of the total operating cost (Demirbas, 2010). Likewise, the biggest challenge for meeting current and future targets in biodiesel production is the limited supply of feedstocks, which necessitates an increase in the efficiency of plant oil production (Durrett *et al.*, 2008; Li *et al.*, 2010). Limitations in biomass quantity may be attributed to environmental and biochemical constraints on net photosynthetic productivity (Schaub & Vetter, 2008). We will discuss specific approaches for increasing biomass supply for biofuel production, such as the selection of feedstocks for biomass production on marginal land (see Section 3.1), genetic improvement in biofuel yield (see Sections 3.2), and utilization of beneficial microorganisms to increase the yield of bioenergy crops (see Sections 3.4).

2.3 Recalcitrance of biomass for biofuel production

Developing non-food, "next-generation" feedstocks such as lignocellulosic biomass has the potential to meet most of the global transportation fuel needs without impacting negatively on food security (Abramson *et al.*, 2010). A major bottleneck for conversion of lignocellulosic biomass to simple sugars (saccharification), to be subsequently converted by microorganisms into ethanol or other products, is the recalcitrance to enzymatic saccharification (Chen & Dixon, 2007; Lionetti *et al.*, 2010). 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 aromatic polymer (Vega-Sanchez & Ronald, 2010). Lignin contributes to biomass recalcitrance and consequently increases the costs associated with conversion (Simmons *et al.*, 2010; Vega-Sanchez & Ronald, 2010). Lignins are complex aromatic biopolymers, consisting of (mainly) syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) units (Simmons *et al.*, 2010). Variations in lignin content and its S-G monomer composition is directly associated with the yield of fermentable sugars (Lee & Voit, 2010). Pectin that embeds the cellulose-hemicellulose network affects the exposure of cellulose to enzymes and consequently the process of saccharification (Lionetti *et al.*, 2010). The lack of efficient biocatalysts and microorganisms to convert lignocellulosic raw materials into liquid fuels is a further bottleneck for sustainable adoption of next-generation feedstocks (Liu & Khosla, 2010). We will discuss several approaches to address the biomass recalcitrance issue, including genetic modification of cell walls (see Section 3.2) and engineering of microorganisms for biomass conversion (see Section 3.3).

2.4 Less-than-ideal physical properties of biofuels

The physical properties of current liquid biofuels including bioalcohol and biodiesel are less-than-ideal for applications in transportation. Although bioethanol currently dominates the biofuel market, some of its inherent physical properties, such as low energy content and

incompatibility with existing fuel distribution and storage infrastructure, limit its economic use (Peralta-Yahya & Keasling, 2010). Biobutanol is a viable alternative to bioethanol because it has a higher energy content and lower solubility in water, can be transported through existing pipelines, and can be used to supplement both gasoline and diesel fuels (Fortman *et al.*, 2008). However, biobutanol has its own shortcomings: it is produced at a lower titer, is much more toxic than ethanol, and requires more energy than ethanol for distillation-based purification from fermentation broth, due to its high boiling-point (Fortman *et al.*, 2008). For example, the energy yield of n-butanol is about half that of ethanol from corn or switchgrass using current acetone-butanol-ethanol (ABE) technology and the low yield increases n-butanol's life-cycle greenhouse gas emission for the same amount of lower heating value (LHV) compared to ethanol (Pfromm *et al.*, 2010). Also, the net energy (6.53 MJ/L) generated during corn-to-biobutanol conversion is greater than that (0.40 MJ/L) of the corn-derived bioethanol (Swana *et al.*, 2011). Although biodiesel obtained from some oil crops, such as *Calophyllum inophyllum*, *Azadirachta indica*, *Terminalia catappa*, *Madhuca indica*, *Pongamia pinnata*, and *Jatropha curcas* oils meet current biodiesel standards in both the European Union (EN 14214) and the United States (ASTM D 6751 02), none of the current biodiesel products can be considered to be the "ideal" alternative that matches all of the key fuel properties that ensure the best diesel engine performance (Pinzi *et al.*, 2009). Plant oils are mostly composed of long-chain (C16 and C18) fatty acids (FAs) such as palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2), and linolenate (18:3), and these FAs differ from each other in terms of acyl chain length and number of double bonds, leading to different physical properties (Durrett *et al.*, 2008). One of the major problems associated with biodiesel properties is the poor flow at low temperatures due to the predominant components of long-chain (C16 and C18) FAs in oil produced from biomass feedstock such as oil seeds and algae (Knothe *et al.*, 2009). For example, the cloud point (i.e., below the cloud point, the formation of crystals clogs the diesel injection) of bio-oil is higher than that of fossil diesel, particularly for oil obtained from some major tropical bioenergy crops such as palm (Abolle *et al.*, 2009a; Abolle *et al.*, 2009b). The presence of saturated methyl esters longer than C12 significantly increases the cloud point, even when blended with conventional diesel fuel (Durrett *et al.*, 2008). Therefore, the current forms of pure biodiesel are not suitable for use in colder climates. We will discuss genetic improvement of biofuel quality as a possible strategy to address the limitations in physical properties of liquid biofuels (see Section 3.2.2).

3. Biological solutions

3.1 Development of new crops for biomass production on marginal lands

To address the two challenges “biofuel versus food competition (Section 2.1)“ and “limited supply of biomass for biofuel production (Section 2.2)”, it is crucial to find ways to produce biomass on marginal lands that are not useful for food production. For many locations around the world, marginal lands represent a valuable resource that could prove to be a viable option for bioenergy crop production. However, crops will need to be tailored to such water-limited and degraded regions, as current biomass crops (e.g., poplar, sugarcane) are poorly suited for biomass production on such lands without irrigation and proper fertilization. Therefore, land-based biofuel crops with high WUE, drought tolerance, and NUE, as well as aquatic biofuel crops, such as microalgae, have great potential for biofuel production on non-agricultural lands.

3.1.1 Land-based biofuel crops with high WUE and drought tolerance

Several emerging or potential bioenergy crops such as *Agave*, sweet sorghum, and *Jatropha* are suitable for production on marginal land because of their high drought tolerance and/or WUE. Succulent species of the genus *Agave* have been cultivated for centuries as sources of alcohol and fibres from rain-fed semi-arid lands. Certain species have been reported to display annual above ground productivities that are comparable to those of the most water-use efficient C3 or C4 crops but with only 20% of the water required for cultivation (Borland *et al.*, 1999). Such characteristics have provoked interest in the potential of *Agave* as a sustainable source of bioenergy feedstock that will not compete with food and fodder production, whilst offering potential for carbon sequestration on marginal and degraded land (Davis *et al.*, 2011). The desirable traits of high productivity and water conservation in *Agave* can be attributed to the operation of crassulacean acid metabolism (CAM) a specialized mode of photosynthetic CO_2 acquisition (Fig. 2). CAM is expressed on a background of Rubisco-mediated CO_2 fixation via the engagement of nocturnal CO_2 uptake catalysed by phosphoenolpyruvate carboxylase (PEPC) and subsequent day-time decarboxylation processes. In CAM plants like *Agave*, stomata open at night when evapo-transpiration rates are low and atmospheric plus respiratory CO_2 is fixed in the cytosol by PEPC. The 3-C substrate phosphoenolpyruvate (PEP) is formed from the glycolytic breakdown of carbohydrates. The final 4-C product, malic acid, is stored in a large central vacuole. During the day, malate exits the vacuole and is decarboxylated through the single or combined action of three enzymes (depending on plant species): NADP malic enzyme (NADP-ME), NAD-ME, and phosphoenolpyruvate carboxykinase (PEPCK). In addition to the 3-C products PEP or pyruvate, CO_2 is released at a high internal partial pressure (pCO_2). This is accompanied by stomatal closure and transpirational water loss is curtailed. By opening their stomata during the cooler night time, CAM plants lose far less water than they would during the warmer day time, and thus *Agave* spp. have lower seasonal water requirements than other bioenergy crops such as corn, sugarcane, Miscanthus, and poplar (Somerville *et al.*, 2010). *Agave* avoids dehydration via structural adaptations such as leaf succulence and shrinkage of the root cortex (hydraulic isolation) can occur at modest soil deficits with cavitation of the root xylem, curtailing water loss from storage tissues to a drying soil (North *et al.*, 2004). Besides having relatively low requirements for water and nutrients, species such as *A. tequilana, A. mapisaga* and *A. salmiana* can provide high yield and high quality biomass for biofuel production. The typically low rates of transpiration in *Agave* leaves obviate the requirement for a highly lignified xylem so lignin contents are relatively low (3–15% by dry weight) whilst cellulose content is relatively high (up to 68%) (Davis *et al.*, 2011). *Agave* biomass can be harvested year-round, producing up to 500 metric tons (green) of biomass per hectare annually (Austin, 2010a; Austin, 2010b). Some *Agave* cultivars possess higher sugar content than sugarcane in Brazil, higher cellulose content than the fastest-growing *Eucalyptus*, and more dry biomass than the genetically-modified poplar trees (Austin, 2010b). Therefore, *Agave* has the potential to become a new bioenergy crop due to its high water use efficiency (3 - 6 fold higher than C4 or C3 plants respectively) (Borland *et al.*, 2009), drought tolerance, high yield, and high quality of biomass. One major limitation in the development of *Agave* into an important biomass feedstock is that there is essentially no genomics-based knowledge to inform improvement strategies for bioenergy purposes. Recently, we initiated an *Agave* genomics project at Oak Ridge National Laboratory (USA) to obtain a genomic and biochemical-based understanding of CAM in *Agave* necessary for its consideration as a biofuel feedstock. Several other *Agave*

transcriptome sequencing projects have been initiated in the United Kingdom (J Hartwell, personal communication) and Mexico (Simpson *et al.*, 2011).

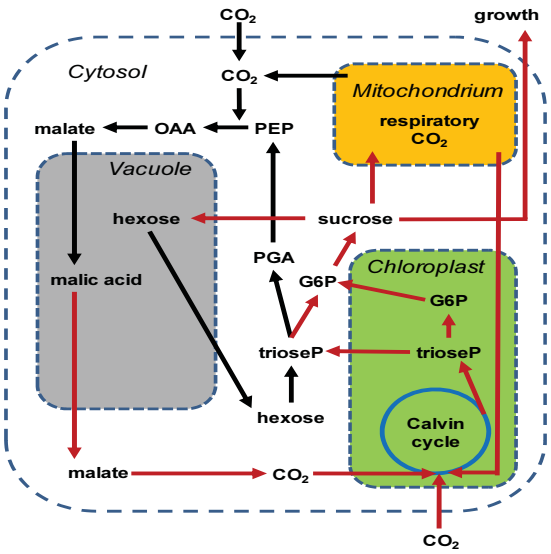


Fig. 2. The CAM pathway. G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetate. Red and black arrows represent light-and dark-period reactions, respectively. Adapted from Holtum et al. (2005), Borland et al. (2009), and Wild et al. (2010).

Sweet sorghum is a potential feedstock for bioalcohol production, with advantages in hot and dry climatic conditions over alternatives, such as sugarcane or maize (Raghuwanshi & Birch, 2010) because it has higher tolerance to salt and drought compared to sugarcane and corn that are currently used for biofuel production. Moreover, the high carbohydrate content of sweet sorghum stalk is similar to sugarcane, but its water and fertilizer requirements are much lower than sugarcane (Almodares & Hadi, 2009).

Jatropha (*J. curcas* L.) has gained much attention for biodiesel production in tropical and sub-tropical countries because of its hardiness, ease of propagation, drought tolerance, high oil content, rapid growth, adaptation to wide agro-climatic conditions, and multiple uses of the plant as a whole (Divakara *et al.*, 2010). Jatropha, known commonly as physic nut, is native or naturalized to parts of Asia, Africa and Central/South America, and has been identified as a multipurpose species with many attributes that give it considerable potential as a biodiesel crop in different parts of the world (Gubitz *et al.*, 1999). It has been shown that the seed kernel of this member of the Euphorbiaceae or spurge family contains 40-60% (w/w) oil deemed unsuitable for cooking due to the presence of toxic esters (Shah *et al.*, 2004). The seed oil of Jatropha was used as a diesel fuel substitute during World War II (Agarwal, 2007), and in more recent years the unmodified Jatropha oil and blends with diesel fuel (Banerji *et al.*, 1985; Jones & Miller, 1991) and transesterified oil esters were tested as an alternative fuel for Thailand (Takeda, 1982; Ishii & Takeuchi, 1987). Despite the growing interest in Jatropha as a biofuels feedstock, it lacks improved germplasm and, until recently, active breeding programs had been lacking. Major germplasm collections for

Jatropha are now found in India (Kaushik *et al.*, 2007; Sunil *et al.*, 2008), Africa, and the Philippines. Information on genetic diversity in Jatropha is still limited since most studies have concentrated on accessions from India where the shrub was brought by the Portuguese. Due to its relatively small genome (2C value of 0.85 pg, in the same size range as that of rice) (Carvalho *et al.*, 2008), Jatropha could become a model woody crop for biodiesel production. Genetic and genomic resources for this emerging biofuels crop are becoming available including a transformation system (Li *et al.*, 2008), a 100x coverage of the *J. curcas* genome sequence (http://www.lifetechnologies.com/news-gallery/press-releases/2010/life-techologies-ad-sg-biofuels-complete-sequece-of-jatropha-geo.html), and a growing library of expressed sequence tags (ESTs) from developing and germinating Jatropha seeds (Costa *et al.*, 2010).

3.1.2 Land-based biofuel crops with high nitrogen use efficiency

Nitrogen use efficiency (NUE) is dependent on many factors including soil nitrogen (N) availability, uptake and assimilation, and carbon-nitrogen flux, and is one of the major limiting factors in increasing crop productivity (Pathak *et al.*, 2008; Raghuram *et al.*, 2008). Although NUE can be calculated a number of ways (Good *et al.*, 2004), a simple yet useful metric is yield per unit of available N in the soil (Kant *et al.*, 2011). Kant and colleagues (2011) suggest that plant N use can be divided into two general stages. The first stage is characterized by N uptake, assimilation into organic compounds (e.g., amino acids), and storage. All of these processes contribute to biomass accumulation. The second stage represents the proportion of N that is allocated to the final yield product (e.g., grain, fruit, and biomass). Relative to traditional agronomic crops, both stages must be considered when assessing next generation bioenergy feedstocks (e.g., lignocellulosic crops). For example, the current land use strategy is to relegate bioenergy crops to marginal lands thereby lessening competition with food crops for limiting arable soils. This would have a direct impact on available N and subsequent plant N uptake and assimilation. In regard to the second stage, lignocellulosic bioenergy crops are often perennial with a biomass yield component. By contrast, traditional agronomic crops are often annual with yield components consisting of grain or fruit. Therefore, allocation within a life-cycle context will be an important component and target for NUE improvement of bioenergy feedstocks. Here, we will discuss NUE in the context of next generation bioenergy crops with a focus on N uptake and assimilation, allocation in a life-cycle and growth habit context, and the interaction of N uptake and allocation driven by genetic controls on root architecture.

3.1.2.1 Nitrogen uptake and assimilation

Stage one of the above NUE model is driven by N uptake and assimilation. In agricultural soils, the predominant form of N is nitrate and to a lesser extent ammonium (Crawford & Forde, 2002). Both high- and low-affinity transporters mediate nitrate uptake and transport. In *Arabidopsis*, for example, there are three main classes of nitrate transporters represented by over 67 genes (Kant *et al.*, 2011). After entering the cell, nitrate is reduced to nitrite by nitrate reductase, and nitrite is further reduced to ammonium in plastids by nitrite reductase (Crawford & Forde, 2002). Ammonium is then assimilated into amino acids through the GOGAT (glutamine synthetase/glutamate synthase) cycle. A number of studies have attempted to increase NUE through the expression of genes associated with N uptake and assimilation. For example, Fraisier et al. (2000) constitutively expressed a high-affinity transporter in *Nicotiana*. Although nitrate influx was enhanced, there was no phenotypic

difference or measurable change in NUE. Similar results were obtained with genetic approaches to alter the expression of nitrate and nitrite reductase N assimilation genes. In these studies, enzyme abundance was increased but complex regulatory feedbacks resulted in no detectable phenotypic improvement (Good *et al.*, 2004). There has been some success with the overexpression of glutamine synthetase, where a 30% increase in kernel number was reported (Hirel *et al.*, 2006). However, no successful commercial lines have been developed using this approach (Kant *et al.*, 2011), which highlights the challenge in transferring laboratory results to field-based applications.

Given that the predominant form of N is nitrate in agriculture soils, we often overlook the potential for organic N source (e.g., amino acids, peptides, etc.) to contribute to overall plant nutritional status. To date, all plant species tested have the ability to acquire amino acids (Lipson & Nasholm, 2001; Nasholm *et al.*, 2009). This includes species that interact with all major mycorrhizal types and non-mycorrhizal types as well. Numerous studies suggest that organic N is an important mineral substrate in the arctic, boreal, temperate, Mediterranean shrubland, and alpine ecosystems (Nasholm *et al.*, 2009). Our understanding of the mechanism by which organic N enters plant cells and is assimilated is quite limited relative to uptake of nitrate and ammonium. There are numerous amino acid transporters belonging to multiple families (Rentsch *et al.*, 2007), yet few have been functionally characterized. Only a handful of studies have investigated how acquired amino acids are assimilated into the N pathway (Schmidt & Stewart, 1999; Thornton & Robinson, 2005; Persson *et al.*, 2006). Based on their results, it appears that amino acids are more likely to be transaminated rather than deaminated and are able to move into shoots. Mycorrhizal associations are known to facilitate proteolysis of soil nitrogenous compounds and enhance the uptake of organic N to plant hosts (see Section 3.4). For sustainable production of bioenergy feedstocks on marginal lands, strategies for increasing NUE though improvement of organic uptake and assimilation should be considered. Possible strategies include a greater understanding and thus modification of the organic N assimilation pathway, and directed plant- microbe interactions (see Section 3.4).

3.1.2.2 Carbon allocation and NUE in annual versus perennial crops

A key challenge for the production of next generation bioenergy feedstocks is increasing yields while maintaining sustainability. As mentioned previously, the existing agricultural concept of NUE relates N uptake to yield (Moll *et al.*, 1982), generally in terms of grain production, and thus has severe limitations in comparing annual to perennial crops. In ecological studies, NUE is associated with whole-plant physiology, the assimilation of N, and other nutrients that are necessary for carbon fixation into sugars and carbon allocation into tissues forming stems, leaves, roots, and leaves. For bioenergy crops, an assessment of the growth habit and life cycle of the crop is necessary in order to compare NUE of seed or oil crops to lignocellulosic energy. In addition, it is clear that NUE should be calculated from harvestable rather than total biomass (Weih *et al.*, 2011). In general, NUE for bioenergy crops is not well studied or characterized, and most studies do not address integration of processes. Whereas annuals depend more on acquired nutrients for growth (Chapin *et al.*, 1990), perennial crops have an advantage with traits such as rapid spring regrowth from existing carbon reserves and generally higher NUE (Jorgensen & Schelde, 2001). Lignocellulosic crops such as poplar, willow, Eucalyptus, and Miscanthus have higher NUE than traditional annual cereal crops in part due to differences in harvest time or multiple year rotation which allow higher rates of translocation of N to storage organs like stems and

roots (Jorgensen & Schelde, 2001). Ecological studies suggest that NUE is the product of mean retention time (MRT), defined as the length of time a unit of N is present in a population, which is representative of N carryover from annual to perennial plant parts (Berendse & Aerts, 1987; Aerts & Chapin, 2000; Weih *et al.*, 2011). Thus, perennials may compensate for lower N acquisition capabilities by having higher N retention due to a lower total biomass turnover rate (Aerts & Chapin, 2000). A high NUE does not necessarily indicate that the system as a whole is more efficient (Jorgensen & Schelde, 2001). One of the criticisms leveled at bioenergy crops is an increased use of N fertilizers derived from fossil fuels and associated greenhouse gas (GHG) emissions (Scharlemann & Laurance, 2008; Erisman *et al.*, 2010). Most of the major industrialized areas of the world, including the United States, European Union, and China have proposed increasing sustainable energy sources through the development of bioenergy crops. However, there have been few discussions over the environmental impacts of changes in the N cycle as a result of increasing biomass production. Thus, improvements in NUE of bioenergy crops will be crucial for mitigation of GHG associated with the production of biofuels (Erisman *et al.*, 2010). NUE of perennial biofuel crops can be improved through a combination of optimizing soil, fertilizer and water interactions, as well as through improvement in traits associated with the physiology of N uptake and assimilation. Development of higher yield bioenergy crops with increased NUE and decreased or neutral soil and atmospheric N losses is critical in order to create a sustainable source of energy for increasing world energy consumption (Erisman *et al.*, 2010).

3.1.2.3 Root architecture

Plants rely on roots and their dynamic architecture for water and nutrient uptake from soil. It is a dilemma, especially under nutrient restricted conditions, for plants to allocate their limited N resources to root growth for foraging of additional nutrients or to shoot development and reproductive structures. Therefore, it is important to understand the changes associated with root growth and development regulated by nutrients especially in the context of nitrogen. Roots have been shown to absorb various forms on N including inorganic nitrate ions and ammonium ions, and organic amino acids, with the help of membrane localized transporters (Nasholm *et al.*, 2009; Masclaux-Daubresse *et al.*, 2010). Nitrogen availability in soil can modify root architecture dynamically. Moreover, the type of N available can also influence root growth (Walch-Liu & Forde, 2008). High nitrate concentrations can reduce primary and lateral root growth, while low nitrate content can enhance outgrowth of laterals (Walch-Liu *et al.*, 2006). Additionally, lateral root development was reduced in *Arabidopsis* plants grown in high sucrose to nitrate ratio (Malamy & Ryan, 2001). Even though high accumulation of nitrates can cause a decrease in root elongation, localized nitrate supply can induce the elongation of lateral roots. In *Arabidopsis*, within species variation was observed in root growth responses as an adaptive mechanism to N availability (Walch-Liu & Forde, 2008). The influence of N content on root growth has been attributed to NRT2.1, a nitrate transporter, although contradicting reports suggest that this protein could act positively or negatively in regulating lateral root growth (Kant *et al.*, 2011). A recent study has revealed a role for the nitrate transporter NRT1.1 in modulating lateral root development under variable nitrate availabilities. This is accomplished by functioning as a plant hormone (auxin) transporter and by regulating auxin accumulation that is necessary for primordia development (Krouk *et al.*, 2010). There are co-localized QTLs for root architectural traits and N uptake traits (Coque *et al.*, 2008).

More studies are needed to dissect the complex interactions between N content regulation, root architectural modifications, and the genetic control of these structural and functional traits associated with nutrient acquisition.

N allocation is a key component related to growth, development, and yield in plants. The N management of plants varies across growth stages. In the early stage, developing shoots and roots act as a sink for N, with assimilated N being used for production of proteins required for structure as well as other regulatory functions (Hirel *et al.*, 2007). At a later stage, roots and shoots serve as a source for N for developing reproductive and storage organs. N remobilization from senescing tissues to young and developing tissues occur at both stages of growth and reproduction (Masclaux-Daubresse *et al.*, 2010). Additional cycling of N can occur through assimilatory and photorespiratory fluxes throughout the life cycle of plants (Hirel *et al.*, 2007). Under high nutrient conditions and at later stages of plant development, root to shoot ratio is low (Garnett *et al.*, 2009). In soils where leaching loss of nutrients are high, root system with dynamic growth is relevant in N uptake, rather than having high root/shoot ratio (Garnett *et al.*, 2009). Under low N conditions, there is a negative relation between root number and yield, possibly due to competition for limiting resources between shoot and root (Hirel *et al.*, 2007). There is variation among species in the involvement of root architecture for N uptake before and after flowering. In some species such as maize, grain yield was correlated to root architecture when grown under low and high levels of N (Garnett *et al.*, 2009). Additional level of regulation comes at the level of nitrate transport components during different stages of root and shoots development, which would directly regulate adaptive responses to various environmental conditions. Root growth and architecture, thus, are important in understanding N uptake efficiency under various soil conditions.

Improving NUE by altering root growth is an important aspect to maximize plant growth and yield. Various aspects of root architecture such as root length, density of lateral root, age of roots, and root hairs can affect N uptake depending on environmental conditions and N availability. Additionally, mycorrhizal and arbuscular microbial associations in plants have also been shown to enhance N uptake (Hawkins *et al.*, 2000; Parniske, 2008). The duration of N uptake is also relevant. Continuance of N uptake through flowering and early grain development was associated with increased NUE in maize (Worku *et al.*, 2007). Deeper roots systems are advantageous in soils where N resources diffuse deep down into the soil profile. Not only the total length, but the root length per volume (root length density) positively correlates with increased NUE, depending on the soil type and species of plants (Garnett *et al.*, 2009). This is due to an increase in root surface area to acquire nutrients from soil, especially in acquiring ammonium ions that are less mobile in soils. However, this is not applicable in soils that have high nutrient content and/or have low leaching, as N levels are saturating and increased surface area due to root hairs is not beneficial (Garnett *et al.*, 2009). A modeling study looking at the relation between N availability and root architecture has shown that the dependence on root morphology in N uptake occurs at low N concentration. Addition of root hairs to the model further reduced the limit of root morphology dependent N concentrations. Moreover, increasing root diameter had no effect on assimilation of nitrate and ammonium ions in the model (Robinson & Rorison, 1983). Within a root system, uptake rates of nitrate ions differ between young and older roots. The older roots could continue to uptake N, even though the rate of uptake might go down, possibly helping improve NUE (Garnett *et al.*, 2009). In an inbred maize line, greater N acquisition was associated with a more responsive root system to low N, a larger and longer root system, and a greater

root/shoot ratio (Liu *et al.*, 2009). Proteolytic enzymes from root exudates can help in degrading proteins, which then can be taken up by plants (Garnett *et al.*, 2009). In parallel, certain factors could negatively affect NUE. Efflux of nitrate and ammonium ions from roots can decrease the net uptake, thereby reducing NUE. In addition, the down-regulation of high affinity N transporters when N is not limited reduces net uptake of N. Environmental factors, such as low light levels and low temperature, limit the net uptake of N (Glass, 2003). Understanding root traits that improve NUE could be used to select plants using breeding or genetic modification techniques for enhanced N utilization capacities.

3.1.3 Aquatic biofuel crops

Biofuels derived from aquatic microbial oxygenic photoautotrophs (AMOPs) including cyanobacteria, algae, and diatoms offer a number of environmental and economic benefits over terrestrial biofuel feedstocks. AMOPs are inherently more efficient solar collectors than terrestrial plants, use less or no land, can be converted to liquid fuels using simpler technologies than those required to break down cellulose, and offer secondary uses that fossil fuels do not provide (Dismukes *et al.*, 2008). Algae in particular have great potential for the renewable production of several bioenergy carriers such as starches for bioalcohols and lipids for biodiesel (Beer *et al.*, 2009). Compared with terrestrial biofuel feedstocks, algae have higher photosynthetic efficiencies for conversion of solar energy into fuels, higher productivities, use of otherwise nonproductive land, reuse and recovery of waste nutrients, less water consumption, use of saline or brackish waters, year-round production, daily harvesting, and reuse of CO_2 from power-plant flue gas or similar sources (Schenk *et al.*, 2008; Beer *et al.*, 2009; Brune *et al.*, 2009; Gouveia & Oliveira, 2009; Posten & Schaub, 2009). The oil yield from microalgae (20,000 to 80,000 liters per acre per year) is 7-31 times greater than the next best terrestrial crop, palm oil (Demirbas & Demirbas, 2011). Among the various microalgae (e.g., *Chlorella vulgaris*, *Spirulina maxima*, *Nannochloropsis* sp., *Neochloris oleoabundans*, *Scenedesmus obliquus* and *Dunaliella tertiolecta*) recently tested, *Neochloris oleoabundans* (fresh water microalga) and *Nannochloropsis* sp. (marine microalga) are suitable for biofuel production due to their high oil content (29.0% and 28.7%, respectively), with a substantial increase (50%) in oil quantity when grown under low nitrogen (Gouveia & Oliveira, 2009). The high productivity of algae suggests that much of the US transportation fuel needs could be met by algal biofuels at a production cost competitive with the cost of petroleum seen during the early part of 2008 (Pienkos & Darzins, 2009). One major limitation is that the current practice used to cultivate, harvest, and process algae for biofuels production is too expensive to make algal biofuel cost-competitive with fossil fuels (van Beilen, 2010).

Cyanobacteria are excellent organisms for biofuel production for a number of reasons: their genomes are relatively easy to manipulate; they are efficient at converting solar energy into biofuels; and they can be grown on non-arable land using photobioreactors (Rittmann, 2008; Liu & Curtiss, 2009; Kumar *et al.*, 2011). An attractive feature of cyanobacteria as a candidate for biofuel-producing microbial systems is that they incorporate the favorable characteristics of both plants and prokaryotics. Unlike the generally utilized biofuel-producing microbes (e.g., *E. coli*, *Z. mobilis*, *S. cerevisiae*, etc.) that have been exploited to make biofuels from glucose produced from polysaccharides through fermentation (Lu, 2010), cyanobacteria can absorb solar energy and fix carbon dioxide (thereby contributing to C sequestration) and are more efficient in converting solar energy and carbon dioxide into useable substrates for biofuels as compared to terrestrial plants. Cyanobacterial cultures can have better water conservation

than terrestrial plant feedstocks, and many cyanobacterial strains are tolerant of marine, brackish, or industrial waste waters, and might effectively utilize water resources that are not suitable for terrestrial crops (Ducat *et al.*, 2011). In general, compared to plants and eukaryotic microalgae, cyanobacteria are more amenable to genetic manipulation for installing biofuel-producing chemical pathways, as demonstrated by the successful reconstruction of metabolic network in *Synechocystis* sp. PCC 6803 (Knoop *et al.*, 2010; Lu, 2010). Cyanobacterial species have been engineered for the production of biofuels (e.g., alcohols, alkanes and hydrogen) (Ducat *et al.*, 2011) and have been tested as a feedstock for biodiesel production by simultaneous extraction and conversion of total lipids (Wahlen *et al.*, 2011). One limitation for biofuel production is that there is inadequate knowledge of cyanobacterial biology and genetic tools in cyanobacteria are less developed in comparison to traditional bioindustrial workhorse organisms, such as *E. coli* and yeast (Ducat *et al.*, 2011).

3.2 Genetic improvement of current bioenergy crops

For sustainable bioenergy production, the crop should be high yielding, fast growing, have low lignin content, and require relatively low energy inputs for its growth and harvest on nonprime agricultural land (Waclawovsky *et al.*, 2010). Genetic engineering can be used to improve bioenergy crops in various aspects such as reducing biomass recalcitrance, enhancing water and nitrogen use efficiency, increasing biofuel yield, and modifying properties of biodiesel. Efficient transformation systems are now available for some biofuel feedstock crops, such as *Camelina sativa* (Lu & Kang, 2008), *J. curcas* (Li *et al.*, 2008; Kumar *et al.*, 2010; Pan *et al.*, 2010), *Panicum virgatum* (Xi *et al.*, 2009), and *Populus* (Song *et al.*, 2006; Cseke *et al.*, 2007; Yang *et al.*, 2009; Yevtushenko & Misra, 2010), making genetic engineering feasible in these crops. Also, genetic diversity in natural or breeding populations has been exploited to develop superior lines for biofuel production. The successful examples of genetic improvement of bioenergy crops are listed in Table 1.

3.2.1 Genetic improvement of biofuels yield

Genes involved in cell wall biogenesis and organization are promising targets for genetic manipulation to overcome the biomass recalcitrance that limits biofuel yields from second generation feedstocks (Yang *et al.*, 2011; Ye *et al.*, 2011). Lignin is one of the most important factors determining cell wall recalcitrance (Simmons *et al.*, 2010; Vanholme *et al.*, 2010). Genetic reduction of lignin content could effectively overcome cell wall recalcitrance to bioconversion, as demonstrated in transgenic alfalfa with down-regulated lignin biosynthetic genes, such as cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), coumaroyl shikimate 3-hydroxylase (C3H), caffeic acid 3-O-methyltransferase (COMT), cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) (Chen & Dixon, 2007; Jackson *et al.*, 2008). Switchgrass (*Panicum virgatum* L.) is a leading dedicated bioenergy feedstock in the United States and down-regulation of the switchgrass COMT gene decreases lignin content modestly, reduces the syringyl:guaiacyl lignin monomer ratio, and consequently increases the ethanol yield by up to 38%, using conventional biomass fermentation processes (Fu *et al.*, 2011). Genetic engineering of biofuel crops with transcription factors involved in the regulation of the phenylpropanoid pathway is another efficient approach to modify lignin biosynthesis. For example, the maize (*Zea mays*) R2R3-MYB factor ZmMYB31 down-regulates several genes involved in the synthesis of monolignols and transgenic *Arabidopsis* plants over-expressing ZmMYB31 show a significantly reduced lignin content with

unaltered polymer composition, and consequently increase cell wall degradability of the transgenic plants (Fornale *et al.*, 2010). An alternative approach to address the lignin issue is to replace monolignols with compounds containing easily cleavable chemical linkages, such as ester and amide bonds, avoiding the undesirable developmental and structural phenotypes associated with the down-regulation of lignin biosynthetic enzymes in transgenic plants (Vega-Sanchez & Ronald, 2010). Inclusion of monolignol substitutes, such as feruloylquinic acid, methyl caffeate, or caffeoylquinic acid with normal monolignols could considerably suppress lignin formation and substantially improve cell wall hydrolysis and fermentation (Grabber *et al.*, 2010).

Besides lignin, hemicellulose (including xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan) also contributes to plant cell wall recalcitrance (Vega-Sanchez & Ronald, 2010). It has been demonstrated that modification of hemicellulose could help overcome biomass recalcitrance. For example, loosening hemicellulose by over-expressing xyloglucanase and xylanase in transgenic poplar accelerates the enzymatic degradation of cellulose in wood (Kaida *et al.*, 2009), and lowering hemicellulose in transgenic poplar by under-expressing *PoGT47C*, a glycosyltransferase gene involved in glucuronoxylan biosynthesis, reduced the recalcitrance of wood to cellulase digestion (Lee *et al.*, 2009). As one of the most abundant polysaccharides on Earth, xylan will provide more than one third of the sugars for lignocellulosic biofuel production when using grass or hardwood feedstocks. Genetic mutations can be generated to remove branches from xylan and consequently simplify lignocellulosic biomass, requiring fewer enzymes for complete hydrolysis (Mortimer *et al.*, 2010). Another possible approach for improving saccharification of plant biomass is to modify pectin in the cell wall. For example, reduction of de-methyl-esterified homogalacturonan (HGA) in both *Arabidopsis* and tobacco plants through the expression of a fungal polygalacturonase (PG) or an inhibitor of pectin methylesterase (PMEI) increased the efficiency of enzymatic saccharification (Lionetti *et al.*, 2010).

Biodiesel is produced by the transesterification of triacylglycerol (TAG) to generate fatty acid methyl esters (FAMEs) (Vega-Sanchez & Ronald, 2010). Biodiesel yield can be improved by genetic manipulation of key genes in the TAG biosynthesis pathway. The final and the only committed step in the biosynthesis of TAG is catalyzed by diacylglycerol acyltransferase (DGAT) enzymes. DGAT is a target for genetic manipulation for enhancing TAG production. For example, expressing a codon-optimized version of a DGAT gene from the soil fungus *Umbelopsis ramanniana* in soybean resulted in 1.5% (by weight) increase in seed oil (Lardizabal *et al.*, 2008). Furthermore, transcription factors that regulate the biosynthetic pathways at the transcriptional level can be utilized for increasing lipid production. For example, two soybean Dof-type transcription factor genes, *GmDof4* and *GmDof11*, enhance lipid content in the seeds of transgenic *Arabidopsis* seeds, indicating that *GmDof* genes may augment the lipid content of soybean seeds by up-regulating genes that are associated with the biosynthesis of fatty acids (Wang *et al.*, 2007). On the other hand, glycerol-3-phosphate supply limits oil accumulation in developing seeds and over-expression of a yeast gene encoding cytosolic glycerol-3-phosphate dehydrogenase (*GPD1*) under the control of a seed-specific promoter resulted in 40% increase in seed oil content in oil-seed rape (*Brassica napus*) (Vigeolas *et al.*, 2007). Although TAG is mainly produced in the seeds of oil crop species, plants can also accumulate small amounts of TAG in the vegetative tissues such as leaves, and leaf TAG levels in the model plant *Arabidopsis* can be increased by up to 20 fold by blocking fatty acid breakdown (Slocombe *et al.*, 2009), expanding the scope of biomass feedstock for biodiesel production. This new route to biodiesel production is further demonstrated by the fact that transferring of an *Arabidopsis*

DGAT gene into tobacco resulted in up to a 20-fold increase in TAG accumulation in tobacco leaves (Andrianov *et al.*, 2010). The full potential of *J. curcas* for biodiesel production is limited by the lack of high yielding varieties with high oil content, and recent research has been conducted to explore existing diversity for yield and oil content by direct selection, hybridization, and creation of diversity by mutation and biotechnological interventions (Divakara *et al.*, 2010).

Directing of photosynthetic carbon partitioning from starch to TAG synthesis may represent a more effective strategy than direct manipulation of the lipid synthesis pathway to increase biodiesel production. For example, inactivation of ADP-glucose pyrophosphorylase in a *Chlamydomonas* starchless mutant led to a 10-fold increase in TAG (Li *et al.*, 2010). The model green alga *Chlamydomonas reinhardtii* accumulates triacylglycerols and forms lipid droplets during nitrogen deprivation, and suppression of the expression of the green algal specific major lipid droplet protein (MLDP) gene using an RNA interference approach led to increased lipid droplet size, but no change in TAG content or metabolism (Moellering & Benning, 2010). Oil harvesting is a major factor limiting the final yield of biodiesel generated from aquatic biomass. To address the harvesting problem in biodiesel production from liquid culture of algae and cyanobacterial, a controllable inducing lysis system, based on integration of bacteriophage-derived lysis genes, into the *Synechocystis* sp. PCC 6803 genome downstream of a nickel-inducible signal transduction system, can be utilized to facilitate extracting lipids for biofuel production. This would consequently eliminate the need for mechanical or chemical cell breakage and facilitate recovery of biofuel from cyanobacteria (Liu & Curtiss, 2009).

3.2.2 Genetic improvement of biofuel quality

As mentioned in Section 2.4, the physical properties of biofuels need to be improved to match the quality of fossil fuels. A lot of research efforts have been devoted to improve the quality of biodiesel. The polyunsaturated fatty acids linoleic acid (18:2) and alpha-linolenic acid (18:3) are major factors affecting the quality of plant oils for biofuels (Lu *et al.*, 2009). Two approaches can be used to address the issue of biodiesel quality. The first approach is to reduce the levels of both saturated and polyunsaturated fatty acids while increasing the amount of monounsaturated fatty acids, such as oleate (C18:1) or palmitoleate (C16:1) (Durrett *et al.*, 2008; Pinzi *et al.*, 2009; Vega-Sanchez & Ronald, 2010). For example, simultaneous down-regulation of two embryo-specific genes in soybean, Delta-12 fatty acid desaturase *FAD2-1* gene and the *FatB* gene encoding a palmitoyl-thioesterase, increased oleic acid levels to greater than 85% compared with less than 18% in wild-type, and lowered saturated fatty acids levels to less than 6% (Buhr *et al.*, 2002). Phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT), encoded by the *Arabidopsis ROD1* gene, is an enzyme for the transfer of 18:1 into the membrane lipid phosphatidylcholine (PC) for desaturation and also for the reverse transfer of 18:2 and 18:3 into the TAG synthesis pathway; and mutation in *ROD1* reduced 18:2 and 18:3 accumulation in seed TAG by 40% (Lu *et al.*, 2009). The second approach is to produce biodiesel comprising medium-chain (C8 and C10) FAs. Currently, *Cuphea* is the only plant source found to produce high levels of medium-chain (C8 and C10) FAs (Fig. 3); and the properties of *Cupea* methyl esters (CuME) meet or exceed the current industrial standard of biodiesel (e.g., CuME displayed a cloud point of -9 to -10°C and a pour point in the range of -21 to -22°C) (Knothe *et al.*, 2009). Understanding the molecular mechanism underlying the accumulation of medium-chain FAs in *Cuphea* and transferring this mechanism to other biomass feedstocks would have great potential for improving biodiesel quality.

Specie	Gene	Biofuels type	References
Arabidopsis thaliana (*Arabidopsis*)	fungal polygalacturonase (PG)	Bioalcohol	(Lionetti *et al.*, 2010)
Arabidopsis thaliana (*Arabidopsis*)	maize R2R3-MYB factor ZmMYB31	Bioalcohol	(Fornale *et al.*, 2010)
Medicago sativa (Alfalfa)	cinnamate 4-hydroxylase (*C4H*)	Bioalcohol	(Chen & Dixon, 2007)
Medicago sativa (Alfalfa)	hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (*HCT*)	Bioalcohol	(Chen & Dixon, 2007)
Medicago sativa (Alfalfa)	coumaroyl shikimate 3-hydroxylase (*C3H*)	Bioalcohol	(Chen & Dixon, 2007)
Medicago sativa (Alfalfa)	caffeic acid 3-O-methyltransferase (*COMT*)	Bioalcohol	(Chen & Dixon, 2007)
Medicago sativa (Alfalfa)	cinnamoyl CoA reductase (*CCR*)	Bioalcohol	(Jackson *et al.*, 2008)
Medicago sativa (Alfalfa)	cinnamyl alcohol dehydrogenase (*CAD*)	Bioalcohol	(Jackson *et al.*, 2008)
Panicum virgatum (Switchgrass)	caffeic acid O-methyltransferase (*COMT*)	Bioalcohol	(Fu *et al.*, 2011)
Populus alba x *tremula* (Poplar)	*PoGT47C* glycosyltransferase	Bioalcohol	(Lee *et al.*, 2009)
Populus (Poplar)	Xyloglucanase (AaXEG2) from *Aspergillus*	Bioalcohol	(Kaida *et al.*, 2009)
Populus (Poplar)	xylanase (HvXYL1)	Bioalcohol	(Kaida *et al.*, 2009)
Populus (Poplar)	Cellulase (AtCel1) from *Arabidopsis*	Bioalcohol	(Kaida *et al.*, 2009)
Zea mays (Corn)	R2R3-MYB factor ZmMYB31	Bioalcohol	(Fornale *et al.*, 2010)
Arabidopsis thaliana (*Arabidopsis*)	Dof-type transcription factor genes, *GmDof4* and *GmDof11* from soybean	Biodiesel	(Wang *et al.*, 2007)
Arabidopsis thaliana (*Arabidopsis*)	ROD1 gene (mutation)	Biodiesel	(Lu *et al.*, 2009)
Brassica napus (Oil-seed rape)	glycerol-3-phosphate dehydrogenase (*GPD1*) gene from yeast	Biodiesel	(Vigeolas *et al.*, 2007)
Glycine max (Soybean)	Delta-12 fatty acid desaturase (*FAD2-1*) and *FatB* gene encoding a palmitoyl-thioesterase	Biodiesel	(Buhr *et al.*, 2002)
Glycine max (Soybean)	Diacylglycerol acyltransferase (*DGAT2A*) gene from the soil fungus	Biodiesel	(Lardizabal *et al.*, 2008)
Nicotiana tabacum (Tobacco)	Diacylglycerol acyltransferase (*DGAT*) gene from *Arabidopsis thaliana*	Biodiesel	(Andrianov *et al.*, 2010)
Chlamydomonas	ADP-glucose pyrophosphorylase	Biodiesel	(Li *et al.*, 2010)
Chlamydomonas reinhardtii (green alga)	Major lipid droplet protein (MLDP)	Biodiesel	(Moellering & Benning, 2010)
Synechocystis sp. PCC 6803	Bacteriophage-derived lysis genes	Biodiesel	(Liu & Curtiss, 2009)

Table 1. Improvement of bioenergy crops using transgenic and mutational approaches.

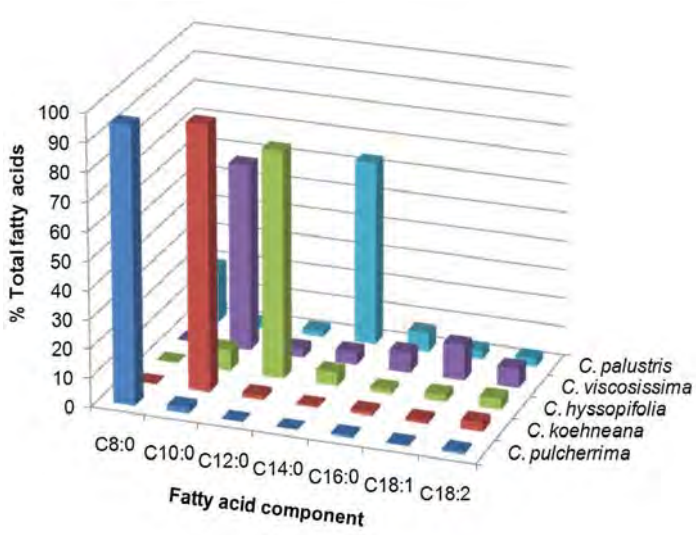


Fig. 3. Variation in fatty acid composition among some *Cuphea* species. Drawn with data from Dehesh (2001) and Knothe et al. (2009).

3.3 Improvement of microorganisms in biomass conversion

3.3.1 Metabolic improvement and genetic engineering of microorganisms for biofuel production

As mentioned in Section 2.3, the lack of efficient microorganisms to convert biomass into liquid fuels is a big challenge in biofuel production using non-food lignocellulosic feedstock which has the potential to meet most of the global transportation fuel needs in sustainable way. The desirable traits of microorganisms for biofuel production include high substrate utilization and processing capacities, fast and deregulated pathways for sugar transport, good tolerance to inhibitors and product, and high metabolic fluxes (Alper & Stephanopoulos, 2009). With beneficial traits for biofuel-related application, some native microorganisms, such as *Clostridium acetobutylicum* for the ABE process, have become the unambiguous organisms of choice for biofuel production in industry (Inui *et al.*, 2008; Alper & Stephanopoulos, 2009; Roberts *et al.*, 2010). However, since the properties required for industrial processing is very different from the features evolved in the native biomes, the transformation from an innate capacity of environmental isolates into an industrially relevant performance can sometimes be strenuous (Alper & Stephanopoulos, 2009). For instance, the current mainstream process of bioethanol production makes use of the basic yeast *Saccharomyces cerevisiae*. This model organism has a proven track record in industrial applications, has superior conversion yields of ethanol from glucose, can tolerate ethanol, and has been the organism of choice for hundreds of years in fermentations to produce wine and other spirits. However, native strains of *S. cerevisiae* have not been exposed to the high concentrations of sugars, aromatic components, and adverse conditions that typically arise in the industrial conversion of lignocellulose to ethanol (Alper & Stephanopoulos, 2009). The same situation exists in the production of butanol using *C. acetobutylicum* that converts acetyl-coA into a mixture of butonal, acetone, and ethanol, and has limited tolerance to the

produced solvents (Alper & Stephanopoulos, 2009; Mao *et al.*, 2010). Despite the difficulties in the utilization of these native microorganisms, which are derived from environmental isolates, the innate capacity of these cells to use recalcitrant substrates is immense. With the advent of modern genetic tools and synthetic biology approaches, we are capable of harnessing the commonly used industrial microorganisms (e.g., *Escherichia coli* and *S. cerevisiae*) for biofuel production (Alper & Stephanopoulos, 2009; Clomburg & Gonzalez, 2010; Sommer *et al.*, 2010). Global transcription machinery engineering, in which transcription factors are adapted to industrial needs by creating mutant libraries and searching for dominant mutations, has proved successful, being able to enhance cellular traits in *E. coli* and yeast species (Liu *et al.*, 2010). Recently, Atsumi et al. (2008) cloned the genes involved in an alternative butanol pathway into *E. coli*, endowing it with the ability to produce reasonable amounts of isobutanol and other alcohols, such as isopropanol. This application, gene transfer along with global transcription machinery engineering, offers the prospect of a desired combination of a high biofuel production and a genetically tractable host. The industrial application of several native and model microorganisms is described as follows.

3.3.2 Industrial application of several representative microorganisms

3.3.2.1 Yeast

As mentioned in the Introduction section, bioethanol is currently the most widely used liquid biofuel, with the global market dominated by Brazil and the United States. The Brazilian system is based on sucrose obtained from sugarcane, which can be converted to bioethanol directly by yeast species without enzymatic pre-treatment, allowing this system to produce an energy surplus estimated at about eightfold (Goldemberg, 2007; Robertson *et al.*, 2008; Argueso *et al.*, 2009). Yeast is a well-established fermenting microorganism in existing commercial-scale ethanol industries. PE-2 is one of the most widely adopted yeast strains for the sugarcane fermentation process, used in about 30% of Brazilian distilleries, generating roughly 10% of the world's bioethanol supply (Argueso *et al.*, 2009). The generation and conversion of fermentable sugars from lignocellulosic materials to ethanol is strongly dependent on the feedstock pretreatment and strain selection (Lau & Dale, 2009). Fermentation of hydrolysates derived from pretreated lignocellulosic biomass is often preceded by washing, nutrient supplementation, and detoxification, which are very costly processes. Recently, a promising technology, known as consolidated bioprocessing (CBP), was developed for biofuel production from lignocellulosic biomass. It involves the use of a single microorganism to convert pretreated lignocellulosic biomass to ethanol by combining cellulase production, cellulose hydrolysis, and sugar fermentation into a single step (Linger *et al.*, 2010; Wen *et al.*, 2010). Although yeast is utilized to ferment sugars derived from cornstarch or sugarcane into ethanol, it cannot ferment the cellodextrins naturally released from lignocellulosic biomass by cellulases and requires multiple enzymes, including β-glucosidases, to quantitatively produce fermentable glucose (Sun & Cheng, 2002; Galazka *et al.*, 2010; Chundawat *et al.*, 2011). Several promising yeast strains have been created, such as 424A(LNH-ST) that exhibits excellent co-fermentation of glucose and xylose (Lau & Dale, 2009). Contrary to yeast, cellulolytic fungi such as *Neurospora crassa* grow well on cellodextrins. Engineering of the *N. crassa* cellodextrin transport system into *S. cerevisiae* promotes efficient growth of this yeast on cellodextrins, and the engineered yeast strains

more rapidly convert cellulose to ethanol when compared with yeast lacking this system in simultaneous fermentation experiments (Galazka *et al.*, 2010). An alternative engineering strategy to construct CBP-enabling yeast species is to endow *S. cerevisiae* with the ability to utilize cellulose by heterologously expressing a functional cellulase system (Wen *et al.*, 2010). Nature has provided two ways of designing such yeast strains: noncomplexed cellulase systems and complexed cellulase systems (i.e., cellulosomes) (Wen *et al.*, 2010; Chundawat *et al.*, 2011). By mimicking the noncomplexed cellulase system, several groups successfully constructed cellulolytic *S. cerevisiae* strains that directly ferment amorphous cellulose to ethanol, although the titer and yield were relatively low (Fujita *et al.*, 2004; Den Haan *et al.*, 2007; Wen *et al.*, 2010). Compared to the noncomplexed cellulase systems, the cellulosome could provide a "quantum leap" in the development of biofuel technology thanks to its highly ordered structural organization that enables enzyme proximity synergy and enzyme-substrate-microbe complex synergy (Bayer *et al.*, 2007). To date, the trifunctional minicellulosomes have been successfully assembled *in vivo* in *S. cerevisiae*, and the resulting recombinant strain could simultaneously hydrolyze and ferment amorphous cellulose to ethanol, providing a relatively convenient engineering platform (Wen *et al.*, 2010).

In the post-genomic era, the availability of rich genomic, proteomic, and metabolomic information provides a solid foundation for yeast strain improvement and engineering. In 1996, the *S. cerevisiae* laboratory strain S288c became the first eukaryote to have its genome completely sequenced (Bayer *et al.*, 2007; Argueso *et al.*, 2009). Since then, other haploid strains from diverse backgrounds have been sequenced (RM11-1a, YJM789, M22, YPS163, and AWRI1631; http://www.broad.mit.edu/), followed by a large-scale effort to determine the genome sequences of many others (Bayer *et al.*, 2007; Wei *et al.*, 2007; Doniger *et al.*, 2008; Argueso *et al.*, 2009). Extensive analyses have been conducted to examine the nucleotide sequence diversity between these strains and the results from these studies provide valuable insights for synthetic biology and artificial biology to create efficient and robust yeast strains.

3.3.2.2 Clostridium

C. thermocellum is a Gram-positive bacterium that is able to ferment cellulose to ethanol, acetic acid, lactic acid, formic acid, hydrogen, and CO_2. As mentioned earlier, *C. thermocellum* is naturally capable of producing butanol. Biobutanol is an attractive fuel as it possesses better energy properties than ethanol, including higher energy content per volume, lower water absorption, and better blending ability. Additionally, *C. thermocellum* appears to be a cellulose-utilizing specialist (Freier *et al.*, 1988; Demain *et al.*, 2005; Tripathi *et al.*, 2010) and produces cellulosome, a multienzyme cellulose-solubilizing complex (Bayer *et al.*, 1985; Bayer *et al.*, 2004; Gold & Martin, 2007; Tripathi *et al.*, 2010). Because of the exemplary capacity of *C. thermocellum* to convert cellulosic biomass without the addition of purified cellulose or hemicellulase enzymes, the CBP platform using *C. thermocellum* provides a promising means for low-cost production of renewable biofuels. Metabolic engineering is required in order to increase the yield of ethanol or other desired products and decrease the rate of mixed-product fermentations carried out by wild type *C. thermocellum*. Unfortunately, reliable genetic tractability has been elusive for *Clostridium* species, in terms of transformation efficiency and screenable genetic marker development (Tripathi *et al.*, 2010). The transformation protocol remains complex and cumbersome in *Clostridium* species, such as *C. acetobutylicum*, *C. perfringens*, *C. septicum*, and *C. thermocellum,* and the efficiency does not compare with that of typical model organisms. When it comes to

the selectable or screenable phenotypes, comprehensive work has been carried out with genetically tractable model organisms, such as *E. coli*, but not in *Clostridium*. Several studies have been performed to transfer these selectable markers into *Clostridium* species. One prominent system transferred to *Clostridium* involves the genes encoding the enzyme orotidine 5-phosphate decarboxylase (PyrF) (Boeke *et al.*, 1984; Haas *et al.*, 1990; Tripathi *et al.*, 2010). Many more studies are undertaken to develop more efficient genetic improvement and engineering approaches for *Clostridium* species.

3.3.2.3 Zymomonas mobilis

Gram-negative fermentative bacterium *Z. mobilis* has been studied for its exceptionally high ethanol production rate and tolerance to the toxicity of the final product and has become a particularly attractive microbial candidate for the CBP platforms (Skotnicki *et al.*, 1983; Linger *et al.*, 2010). *Z. mobilis* is capable of fermenting sugars at low pH and has a naturally high tolerance to many inhibitory compounds existing in hydrolysates derived from lignocellulosic biomass (Zhang *et al.*, 1995; Linger *et al.*, 2010). Additionally, the Entner-Doudoroff pathway naturally existed in *Z. mobilis* allows it to reach the near-theoretical maximum ethanol yields during fermentation while achieving relatively low biomass formation (Swings & De Ley, 1977; Linger *et al.*, 2010). To establish *Z. mobilis* as a CBP host, a necessary prerequisite is that *Z. mobilis* must have high levels of cellulolytic enzyme expression. However, achieving high-level expression of cellulases is not the only hurdle to overcome. It is imperative that these enzymes must be translocated to the extracellular space and contact the lignocellulosic substrate directly (Linger *et al.*, 2010). The most obvious means to achieve this translocation is by harnessing the host's protein secretion apparatus. It has been reported that several *Z. mobilis* strains natively produce an endogenous activity against carboxymethyl cellulose and that this activity can be detected extracellularly, which can be adapted to secrete cellulolytic enzymes (Linger *et al.*, 2010). All these results suggest that *Z. mobilis* may be adept at producing cellulases, and as this attribute is essential for an industrial application, *Z. mobilis* serves as an ideal candidate for CBP. To date, *Z. mobilis* has shown successful records in CBP and has been successfully engineered to ferment the pentose (C_5) sugars, xylose, and arabinose (Zhang *et al.*, 1995; Deanda *et al.*, 1996; Linger *et al.*, 2010).

3.3.2.4 Trichoderma reesei

T. reesei (syn. *Hypocrea jecorina*) is a mesophilic soft-rot ascomycete fungus (Mandels & Reese, 1957; Martinez *et al.*, 2008). This biomass-degrading fungus represents a paradigm for the production bioethanol and a range of key biochemical building blocks, such as aspartic acid, glucaric acid, glutamic acid, glycerol, sorbitol, and hydroxybutyrolactone, because it naturally possesses enzymes that hydrolyze lignocellulosic polysaccharides (Martinez *et al.*, 2008; Alper & Stephanopoulos, 2009). It has enjoyed a long history of safe use in industrial enzyme production and is currently widely used as a source of cellulases and hemicellulases for the hydrolysis of plant cell wall polysaccharides (Nevalainen *et al.*, 1994; Martinez *et al.*, 2008). Although genetic engineering techniques, gene knockout protocols, and DNA-mediated transformation systems have improved the performance of industrial *T. reesei* strains (Martinez *et al.*, 2008), further studies are needed to expand its extraordinary potential for biofuel production.

3.4 Utilization of beneficial microorganisms to increase the yield of bioenergy crops

All plant associated microenvironments, especially the rhizosphere, are colonized by the microbes in high abundance (Berg *et al.*, 2005). Soil microorganisms including bacteria and

mycorrhizal fungi promote plant growth either directly by acting as biofertilizers, phytostimulators, rhizoremediators or indirectly as biocontrol agents. The controlled use of microbes has emerged as a promising solution for the sustainable production of agronomically important crops. This is important as the production of bioenergy feedstocks has the potential to place additional burden to already constrained natural resources such as land, water and nutrients. In this section we discuss the how the partnerships between plants and their microbial associates can be used to bolster biomass production of bioenergy feedstocks in an environmentally conscious fashion.

The population density of the bacteria in the plant rhizosphere is high, with estimates ranging from 10^5-10^7 CFU g^{-1} fresh weight of bacteria (Bais *et al.*, 2006). Although rhizobacteria may be neutral or antagonistic to host plant growth and productivity, most (about two thirds) are reputed as beneficial (Furnkranz *et al.*, 2009). This has been demonstrated in several studies with rhizobacteria. For example, differ isolates of *Methylobactrium* have been shown to improve germination, growth and yield of sugarcane (Madhaiyan *et al.*, 2005), and *Enterobactor sp.638* has been shown to have a pronounced influence on growth and development of poplar cuttings in marginal soils (van der Lelie *et al.*, 2009). As described earlier (Section 2.1), one way of avoiding competition between food and bioenergy crops is to modify bioenergy feedstocks for growth on marginal lands. These marginal lands are comprised of soil that lacks one or more essential nutrient, are water limited or are contaminated by pollutants such as heavy metals. Plant associated bacteria can be used for the economic production of biofuels by enabling the cultivation of bioenergy crops on these otherwise unsuitable marginal lands. For example, several greenhouse and field studies have demonstrated the efficiency of non-nodule forming nitrogen fixing bacteria on different host plant species including sugarcane, soybean and rice (Boddey *et al.*, 1995; Mano & Morisaki, 2008; Mishra *et al.*, 2009). In switchgrass, inoculation of the seedlings by a consortium of different rhizosphere microbes increased N-uptake up to 6-fold (Brejda *et al.*, 1998). In poplar and willow, there is a role for endophytes in fixing atmospheric nitrogen (Doty *et al.*, 2009). Several genera of bacteria including *Bacillus, Enterobactor, Pseudomonas* and *Azotobactor* have been shown to mineralize or solubilize phosphate in the rhizosphere making it available to the plant (Vassilev *et al.*, 2006 and references therein).

The ability by which plants acclimate and tolerate abiotic stress can be enhanced by their microbial associates. With plant-rhizobacteria interactions, for example, the bacteria produce compounds including phytohormones (e.g., auxin and ethylene), which in turn modulates plant growth and can improve host plant stress tolerance and fitness. The bacteria *Azotobactor* and *Azospirillium* were originally thought to improve host plant growth through fixed nitrogen, but additional studies have identified multiple mechanisms including the production of hormones such as IAA, Gibberellins, and cytokinins (Okon *et al.*, 1998). Many root associated bacteria are known to produce auxin derivatives (e.g., Indole-3-acetic acid) and such bacteria can modify root architecture, which in turn influences water and nutrient uptake (see Section 3.1.2). In poplar, inoculation of rooted cuttings, with auxin-producing endophytic bacteria improved growth by up to 60% (Taghavi *et al.*, 2009). Rhizobacteria also modulate ethylene levels in plants either through the auxin they produce or with the activity of bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Bactria possessing this enzyme can use ACC as an immediate precursor of ethylene, thereby reducing plant ethylene levels that leads to increased root growth. This is important given that ethylene plays a key role in stress signal transduction pathways. In addition to auxin, ethylene and

gibberellin producing bacteria have been isolated from pine (Bent *et al.*, 2001), rapeseed (Noel *et al.*, 1996), lettuce (Noel *et al.*, 1996), and soybean (Garcia de Salamone *et al.*, 2001). Some of these bacteria stimulate plant growth by gibberellin biosynthesis (Gutierrez Manero *et al.*, 2001). Although our current understanding of the role of soil bacteria in improving host plant abiotic stress tolerance is limited, a few studies have shown some promise with bioenergy feedstocks using this approach. One notable example is from Ye *et al.* (2005), where inoculation of *Miscanthus* with a consortium of soil bacterial enhanced tolerance to salinity.

Some bioenergy feedstocks such as poplar and willow have been used for remediation of groundwater and soil contaminants such as BTEX (benzene, toluene, ethylbenzene and the xylene isomers), TCE (trichloroethylene), and diesel. In poplar, selective enrichment of the *rhizospheric* and endophytic bacteria has been observed in the presence of the contaminants (Barac *et al.*, 2009). Use of recombinant bacteria modified to contain specific degradation pathways has emerged as a novel tool for growing plants on the contaminated soil (van der Lelie *et al.*, 2009). Inorganic pollutants such as heavy metals induce oxidative stress by enhancing ethylene production which in turn reduces biomass productivity (Arshad *et al.*, 2007). Inoculation of plants with bacteria harboring ACC deaminase can be used to enhance plant growth and improve metal tolerance. However, further experimentation is required to exciting test this possibility.

In addition to their role in plant nutrition and rhizoremediation, management of plant-microbe interactions can be used in low-cost integrated disease management strategies. Many soil bacteria produce anti-microbial compounds which prevent the growth of harmful soil born fungi. This strategy has shown some promise in bioenergy corps. For instance, in Eucalyptus, a strain of *Pseudomonas fulva* has been shown to reduce *Cylindrocladium candelabrum* growth by 33%, which causes mini-cutting rot in Eucalyptus and several other tree species. A study by Fucikovsky *et al.* (2006) has shown some promise for this approach in controlling bacterial infection of *Agave*, an emerging bioenergy feedstock plant. In addition to their anti-microbial activity, soil microbes and endophytes have also been used to activate plants defense systems against pathogens and herbivory. This phenomenon known as induced systemic resistance (ISR) is largely dependent on the ethylene and jasmonic acid signaling in the plant (van Loon, 2007). On the microbial side, several compounds secreted by the soil bacteria such as, salicylic acid, Acyl homoserin lactones, acetoin, and 2,3-butanediol have been shown to induce ISR (Ryu *et al.*, 2003; Shuhegge *et al.*, 2006; van Loon, 2007). Interestingly, unlike other biocontrol associations ISR does not require an extensive colonization of the host plant (Kamilova *et al.*, 2005). However, due to the complexity of the bacterial communities in the soil, a more comprehensive understanding of their genomes and secretomes is necessary before we further explore the use of soil bacteria as biocontrol agents.

The mycorrhizal symbiosis between soil fungi and plant roots represents the most widespread association between plants and microbes. Mycorrhizal symbioses are prevalent in all major terrestrial biomes (Smith *et al.*, 1997). Currently we face many global challenges to our energy supply (see Section 2), and soil functioning through plant-mycorrhiza interactions could play an important role in helping us address these challenges. Specially, plant-mycorrhiza interactions may 1) enhance carbon sequestration in terrestrial ecosystems to stabilize the atmospheric CO_2 concentration, 2) increase the production of food and bioenergy crops by increasing nutrient availability, 3) remediate degraded, polluted or desertified soils, and 4) develop sustainable cropping systems aimed at improving WUE and

soil properties to minimize erosion, water pollution, and eutrophication (Schreiner *et al.*, 2003). All of these aspects make plant-mycorrhiza interactions an excellent approach for improving the sustainability of bioenergy feedstock productivity.

Mycorrhizal fungi are an important soil carbon sink and often constitute 20-30% of total soil microbial biomass (Leake *et al.*, 2004). They can reduce soil carbon loss by immobilizing carbon in their mycelium, by extending root lifespan, and by improving carbon sequestration in soil aggregates (Langley *et al.*, 2006; Rillig & Mummey, 2006). Bacteria and fungi play distinct roles because of their inherent stoichiometry, especially of C and N. The average C : N ratio in bacteria is about 4 and in fungi about 10, and fungi generally respire less, resulting in higher carbon use efficiency (CUE) relative to bacteria (Six *et al.*, 2006). Recent studies, however, found considerable overlap in CUE-values of bacteria and fungi that is dependent on a number of factors including species and functional group identity, quantity and quality of substrates, and abiotic factors (Six *et al.*, 2006). Mycorrhizal fungi may have higher CUE than saprophytic fungi and bacteria (Wallander *et al.*, 2003). Further, fungal mycelia are more recalcitrant in soil relative to bacteria. Mycelia are comprised of complex nutrient-poor carbon forms such as chitin and melanin, while bacterial membranes mainly consist of phospholipids that are quickly re-assimilated by soil biota. Although, the mechanisms of microbial contribution to soil organic carbon sequestration are poorly understood *in situ*, an overall increase in fungal-dominance is typically associated with high organic-matter content and low substrate quality, i.e. high C:N ratio (Bardgett, 2005; van der Heijden *et al.*, 2008). The effect of mycorrhizal fungi on soil carbon sequestration may be highly specific to the combination of plant and symbiont species (Kiers & van der Heijden, 2006) and soil fertility (Allen *et al.*, 2003). These underlying traits need further elucidation, yet it appears that across ecosystems, different types of mycorrhizal fungi prevail and are related to particular plant traits and growth limiting nutrients (Cornelissen *et al.*, 2001; Read & Perez-Moreno, 2003).

So far, mycorrhizal application has shown a substantial increase in the yield properties such as aboveground biomass (Sramek *et al.*, 2000). Although no clear mechanism other than an improvement in the nutritional status has been proposed (Toussaint, 2007), beneficial fungus–plant interactions has shown enhancement in productivity of crops by synthesizing a number of active compounds such as alkaloids, oils, resins, tannins, natural rubber, gums, waxes, dyes, flavors and fragrances, pharmaceuticals, and pesticides (Rai *et al.*, 2001). For example, the suitable selection of host plant–fungus genotype led to an altered accumulation of essential oil levels in arbuscular mycorrhiza-colonized plants of *Mentha arvensis* (Freitas *et al.*, 2004) and sweet basil *Ocimum basilicum L.* (Copetta *et al.*, 2006; Copetta *et al.*, 2007; Toussaint, 2007).

Colonization with mycorrhizal fungi results in improvements in plant fitness and nutrition (Smith *et al.*, 1997). The network of extrametrical hyphae facilitate acquisition and transport of many ions to roots, particularly mobile ions such as P, N, K, S, CA, and Zn. In addition, mycorrhizal fungi enhance the reabsorption of nutrients lost through root exudation and contribute to the soil fertility (Hamel, 2004; Rillig, 2004). A functional specialization is recognized according to the type of the mycorrhizal fungi, arbuscular mycorrhiza (AM) or ectomycorrhiza (EM). The most important function of AM for plant growth is increasing uptake of P. There has been strong evidence that supports the role of AM mycelia in mineralization and uptake of organic P (Tarafdar & Marschner, 1994; Koide & Kabir, 2000). The rapid linear extension rates and narrow diameters of AM hyphal networks along with the wall-bound extracellular phosphatase enzymes (Joner *et al.*, 2000) enable the enzymes to

reach in soil pores that are otherwise inaccessible due to their small size and distance from the root. It is well established that many EM fungi are active producers of phytase and phosphatase enzymes (Leake & Read, 1997), and some can obtain both P and N from a range of organic sources, including partially decayed tree litter, pollen, and nematodes (Read & Perez-Moreno, 2003). In soil microcosms, between 35% and 40% of the total P content of partially decayed tree litter was removed by colonizing EM mycelium, with the majority of this P being mobilized from organic compounds. In the absence of EM mycelium, moist and non-sterile partially decayed tree litter releases inorganic P slowly (Bending & Read, 1995). It was reported that 15% of P and 12% of N supplied to trees in boreal forest ecosystems may come from EM derived associations (Read & Perez-Moreno, 2003). Furthermore, some EM fungi are toxic to fungal-feeding micro-arthropods such as collembola and significant amounts of N can be obtained by mycorrhizal fungi digesting of dead collembolan (Klironomos & Hart, 2001). In addition, mycorrhizal fungi appear to be able to acquire P from a range of inorganic P sources, including some calcium and aluminium phosphates that have extremely low solubility (Yao *et al.*, 2001), but it is not known whether the fungi are directly involved in their solubilization. Uptake of insoluble P sources by AM may be facilitated by P-solubilizing bacteria, and there may be mutualistic interactions between these two groups of organisms (Villegas & Fortin, 2001). EM mycelia have also been shown to obtain P from a range of sparingly soluble mineral sources such as aluminium phosphate (Cumming & Weinstein, 1990), and their production of organic chelators such as citric and oxalic acids, together with hydroxamate siderophores, are implicated in major mineral weathering processes and podsolization (van Breemen *et al.*, 2000). These findings are of importance for biogeochemistry and processes of soil maturation. Besides their roles in P nutrition, both AM and EM fungi play a major role in the uptake of N by plants. Based on the studies of monoxenic fungal cultures, AM mycelium has been shown to have a role in the uptake of ammonium, nitrate, glycine, and glutamine. AM fungi increase decomposition and subsequent capture of inorganic N from complex organic materials such as plant litter (Hodge *et al.*, 2001). These kind of responses have been considered characteristic of EM but not AM fungi (Leake & Read, 1997). Furthermore, ectomycorrhizal fungi have high-affinity amino acid uptake systems (Wallenda *et al.*, 2000) and highly developed proteolytic capabilities enabling them to directly access macromolecular N (Abuzinadah & Read, 1989). Although use of mycorrhizal fungi for improving crop production has been limited to medicine or food production, studies are ongoing to explore their roles in bioenergy production.

4. Conclusion and perspectives

Declining availability and political instability in the supply of fossil fuels has focused efforts on developing liquid biofuels to meet our ever-increasing energy requirements. However, a huge gap remains between biofuel production and future energy needs, as reflected by the fact that current biomass generated on agricultural lands cannot support sustainable biofuel production, and the physical properties of both bioethanol and biodiesel are less than ideal for application in transportation. In this chapter, we have described four major challenges in sustainable biofuel production and discussed biological innovations for solving these challenges. Currently, biofuels are commercially produced mostly from the so-called first generation bioenergy biomass (e.g., corn and soybean), and worldwide efforts have been undertaken to realize the potential of next-generation bioenergy crops (e.g., switchgrass,

Populus, Jatropha, and algae). With the availability of increasing numbers of sequenced plant genomes (http://www.phytozome.net/) across a large evolutionary space, a better understanding of the gene networks regulating the biological pathways relevant to biomass composition, productivity and resource use efficiency will be obtained. Such knowledge can subsequently be exploited to design effective strategies for the genetic improvement of bioenergy crops that will include overcoming the recalcitrance of lignocellulose to enzymatic saccharification.

CAM species such as *Agave* show considerable promise as a biofuel crop for the future due to their high water-use efficiency, tolerance to abiotic stress (e.g., drought and high temperatures), and potential for high biomass production on marginal lands (Borland *et al.*, 2009; Jaradat, 2010; Somerville *et al.*, 2010). Further research is needed to establish the relationship between CAM and nutrient uptake and assimilation in order to further enhance the significance of using *Agave* as a biofuel feedstock. Reported discrepancies on how the water-conserving CAM pathway impacts on the use and allocation of N need to be resolved in order to fully exploit the sustainable farming of *Agave* for biomass by reducing dependence on commercial nutrients, minimising the cost of production and diminishing environmental pollution.

The newly-developed synthetic biology (i.e., the ability to design and chemically synthesize genetic sequences imported into host cells) could expand our capacity to construct and improve pathway performance, enabling diversification of the biofuel-type molecules produced in standard model organisms (Alper & Stephanopoulos, 2009). For producing biofuels identical or similar to petroleum-derived transportation fuels, synthetic approaches have been used to engineer microbes to synthesize biofuels, such as butanol and fatty acid- or isoprenoid-based fuels, which are nearly identical to gasoline and diesel (Ghim *et al.*, 2010). Furthermore, the recent introduction of artificial biology, fuelled by the capacity to synthesize large pieces of DNA, has made it possible to construct cellular systems *de novo* (Alper & Stephanopoulos, 2009; Biello & Harmon, 2010; Bornscheuer, 2010; Noskov *et al.*, 2011) and thus has created a new efficient strategy for sustainable production of biofuels with ideal quality and in commercial quantities.

A better understanding of the soil microorganisms and their interactions with the host plants in their ecosystem will ensure an opportunity for the use of bacteria and mycorrhizal fungi to enhanced sustainable bioenergy crops production. Thus, in properly managed agriculture systems, microbial symbioses can act as biofertilizer, biocontrol agent, and soil improver, likely being one of the key solutions to the problems associated with sustainable biofuel production. Recent genome sequencing efforts for the plant associated microbes have been increasing our knowledge about these organisms and the way they interact with the plants (Martin *et al.*, 2008; Taghavi *et al.*, 2009). We still need to find better ways to inoculate and identify suitable vectors for introducing these beneficial microbes in the plant ecosystem. The increasing amount of genomic data and the systems biology studies will help us find the most suitable consortia of microbes for inoculation in the coming years.

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