Chilling tolerance and biomass production of *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes) in cool climatic conditions of northern Japan

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2019

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Chapter 1: General Introduction

Background of the present study

With the changing scenario of modern day civilization, energy availability is increasingly becoming a crucial issue. Currently most of the developed nations rely on fossil fuel sources for energy, which are non-renewable, limited in supply and convert the fossilized carbon reserves into carbon di oxide, which acts as a greenhouse gas, responsible for global warming. But in the future, we will be needing energy sources, that are renewable and sustainable, efficient and cost-effective, convenient and safe (Chum & Overend, 2001; Mckendry, 2002). The first sign of an alternate and more sustainable source of energy dates back to 1970s, when America faced the first fossil oil crisis, which resulted in a spike in oil prices that led to the first push for the development of renewable energy needs (Gent et al., 2017). Biomass is the most common form of renewable resource that is abundantly used in the developing nations but not so much in the industrially developed nations (Mckendry, 2002). In 1992 at the Rio United Nations Conference on environment and development, the renewable intensive global energy scenario (RIGES) suggested that, by 2050, approximately 50% of the world's current primary energy consumption, could be met by biomass and 60% of the world electricity market would be supplied by renewables sources of which biomass is a significant part (Mckendry, 2002).

Biomass is a term for all organic materials that are produced by plants on a renewable basis (Catchpole & Wheeler, 1992). It is produced by green plants that convert sunlight into plant material through photosynthesis (Catchpole & Wheeler, 1992; Mckendry, 2002). Biomass is an indigenous energy source, available in most countries and its diversification will lead to more secure energy supply. Biomass is an important contributor to the world economy. Apart from energy production, about 60% of the needed process energy in pulp, paper and forest products is

supplied by biomass (Chum & Overend, 2001). Biomass production can generate employment and it has environmental benefits, such as reduced emissions, reduced leaching of fertilizers and reduced use of pesticides (Mckendry, 2002). The term biomass energy can refer to any source of heat energy produced from non-fossil fuel origin like crop residues (haulms of grain legumes, stalks of maize, sorghum and millets, straw from rice, wheat, barley and oat), energy crops, timber from forests, animal waste and municipal waste (Catchpole & Wheeler, 1992; Fischer & Schrattenholzer, 2001; Field et al., 2008). The search for a sustainable substitute for fossil fuel has stimulated research into bioenergy crops (Purdy et al., 2013).

As pointed out previously, any crop can be considered as a source of biomass. However, a 'dedicated bioenergy crop' refers to nonfood crops that are solely grown for biomass production (Gent et al., 2017). For a dedicated energy crop the following criteria must be fulfilled, the feedstock must: 1) Be easily and reliably transformed in useful forms of energy; 2) Have dense tillering; 3) Have high energy per unit of dry matter; 4) Be available throughout the year; 5) Favor the cost of production and delivery; 6) Be a source of renewable energy; 7) Be tolerant to biotic and abiotic stress; 8) Not compete with the arable crop production; 9) Environmentally secure (Mckendry, 2002; Purdy et al., 2013; Matsuoka et al., 2014). Major biomass energy crops may include sorghum, maize, reed canary grass, *Miscanthus* spp., sugarcane (Sims et al., 2006), switchgrass and newly developed miscane (Sacks et al., 2013). It is very crucial to select an appropriate energy crop, for best adaptation, benefit and restoration of degraded land (Mckendry, 2002).

Sugarcane

Sugarcane is a large perennial tropical and subtropical grass, adapted to environments with warm temperatures, abundant sunlight and water and year round cropping season. This warm-climate

adaptation thus limits sugarcane's cultivation range between 31º N and S of the equator (Moore et al., 2013). Because of its high biomass production capacity (~1.9 billion tons worldwide) and being a principal source of sugar ethanol, this crop is cultivated in approximately 100 countries on 26.7 million-ha of land (Moore et al., 2013; FAOSTAT, 2016).

According to the current classification, genus *Saccharum* includes six species, *Saccharum officianrum* L., *S. spontaneum* L., *S. sinense* (Roxb) Jesw., *S. barberi* Jesw., *S. robustum* Brandes & Jeswiet ex Grassl. and *S. edule* (Hussk.), among which *Saccharum spontaneum* L. and *Saccharum robustum* Brandes and Jewiet ex Grassl are considered wild species and rest are cultivated (Moore et al., 2013; Paterson et al., 2013). Today's cultivated sugarcane species are hybrids between *S. officianrum*, the noble cane, *S. spontaneum* and contribution from other species including *S. sinense*, *S. robustum* and *S. barberi* and even from other related genera like *Miscanthus*, *Erianthus*, *Narenga*, *Impereta*, *Eriochrysis*, *Eccolipus*, *Spodiopogon* and *Sclerostachya* (Barber, 1920; Parthasarathy, 1948; Brandes, 1956; Clayton, 1972; Clayton, 1973; Moore et al., 2013; Paterson et al., 2013). Sugarcane and its related genera are classified into an informal taxonomic group called '*Saccharum* complex' (Mukherjee, 1954; Daniels & Daniels, 1975) which has proven to become an important benchmark for genetic improvement of sugarcane by later breeders (Paterson et al., 2013). These improvement aspects include vigour, abiotic stress resistance, ratooning ability and disease resistance (Berding & Roach, 1987; Paterson et al., 2013). Breeders have tried to cross *Miscanthus* and *Erianthus* with sugarcane to improve its agronomic characteristics, however, morphology based identification has proven to be difficult in selection and breeding (Paterson et al., 2013). But recently, molecular genomics have given opportunity to identify and select crosses at seedling level and follow the introgressed traits in later generations (D'Hont et al., 1995; Alix et al., 1999; Piperidis et al., 2000).

Cold sensitivity of sugarcane

C_4 plants are tropical in origin and are considered generally as cold-sensitive (Berry & Bjorkman, 1980). This susceptibility to cooler temperatures is attributed to higher temperature optima of key enzymes like phosphoenolpyruvate carboxylase (PEPc) (Treharne & Cooper, 1969) along with cold-lability of pyruvate orthophosphate dikinase (PPDK) (Uedan & Sugiyama, 1976; Sugiyama et al., 1979; Edwards et al., 1985). Sugarcane belongs to the cold-sensitive NADP-ME C_4 subtype (Sugiyama et al., 1979; Edwards et al., 1985). The optimum growth temperature for sugarcane is assumed to be around 35°C (Khan et al., 2013). In general, cold injury in sugarcane depends on three factors, tissue type, exposure temperature and time of exposure (Arceneaux et al., 1951). Commercial sugarcane (*Saccharum officinarum* hybrids) is very cold sensitive and shows a severe decrease in photosynthetic rate after short term exposure to chilling (Głowacka et al., 2016). When grown at 10/5 ºC, sugarcane shows a reduction of >98% in photosynthetic rate, in comparison to when grown under normal temperatures (25/20 ºC) (Głowacka et al., 2014). Jain et al. (2007) found that temperatures of 15 and 6 ºC can reduce stubble bud sprouting to 23 and 56 percent respectively compared to when grown under optimum temperatures. Leaves and buds get damaged at a temperature of -2.7 to -3.9 ºC while stem damage occurs at -3.3 to -5 ºC (Irvine, 1978; Tai & Miller, 1996). Underground buds get damaged where soil reaches freezing temperatures during winter (Burner et al., 2017). Terminal buds starts to get damaged at a temperature of 0 to -2.2 ºC, apical and auxiliary buds are killed at -2.8 to -3.9 ºC. Further decrease in temperature leads to weeping of lateral buds followed by cracking of stalks at a temperature of -5.6 ºC (Legendre et al., 2013; Hale et al., 2016). In the US Louisiana (about 31 ºN) is the northern limit of commercial production of sugarcane (Matherne et al., 1977; Richard Jr et al., 2008) beyond which the crop fails to ratoon following winter (Stokes et al.,1961). Hence, ratoon cold tolerance is necessary for

maintaining yield throughout the growing period of the crop (Burner et al., 2015). Du et al. (1999a) has shown that some of the subtropical sugarcane hybrids show more cold tolerance than tropical species.

Breeding for cold tolerance in sugarcane

Although sugarcane is considered as a cold-susceptible crop (Głowacka et al., 2016), studies show that there are varying degree of response to cold among different varieties of sugarcane (Du et al., 1999a; Hale et al., 2014), which is indicative of the potential for selective breeding in sugarcane to improve cold tolerance (Legendre et al., 1986; Du et al., 1999a). In order to succeed under cooler environments sugarcane stalk buds must survive under sub-zero temperatures (Hale, Viator, Kimbeng, & Veremis, 2017). Breeding sugarcane genotypes for cold tolerance can expand its production area beyond current cultivation zone (Cobill, 2007).

The polyploidy nature and interspecific hybrid characters make sugarcane highly heterozygous (Lu et al., 1994; D'Hont et al., 1996). Transmission of cold tolerance from parental genotypes to offspring in sugarcane was demonstrated by Breaux and Irvine in 1976. Many of the related species of sugarcane had been tried to introgress cold tolerance in sugarcane.

Saccharum spontaneum

Saccharum spontaneum, a wild subtropical species of sugarcane with robust rhizomes and profuse tillering ability, has contributed in improving abiotic stress tolerance (cold and drought) and improved perenniality of cultivated sugarcane (Panje, 1972). *Saccharum spontaneum* is among the most primitive of sugarcane species with a center of diversity in India and a distribution through tropical and subtropical parts of the world (Ming et al., 2006). It is distinguished by its thin stalks and high fiber content. *S. spontaneum* is considered to having the highest variability within

Saccharum genus (Mary, Nair, Chaturvedi, & Selvi, 2006). 10-20% of chromosomes of modern day cultivars of commercial sugarcane are derived from *S. spontaneum* (D'Hont et al., 1996).

As early as in 1940s Brandes identified *S. spontaneum* clones that can survive at a temperature below -30 °C for 18 days. However, hybrids between them and commercial sugarcane could not survive at a temperature of -4 ºC. Recently Hale et al. (2013 and 2014) had demonstrated a range of cold tolerance ability among *Saccharum* accessions grown in USDA-ARS Sugarcane Research Unit (SRU) in northern Louisiana. Hybrids with alleles from cold-tolerant *S. spontaneum* were seen to increase tolerance to cold stress, where F_1 populations of unselected hybrids between commercial cultivars × *S. spontaneum* has shown adequate levels of freeze tolerance (Legendre & Burner, 1995). *S. spontaneum* is very well adapted to a varied environmental conditions including some harsh climates (Roach, 1987; Ming et al., 2006). Due to its high variability in cold tolerance, its germplasm is often used in breeding to introgress cold tolerance among sugarcane in several countries worldwide including the US, Brazil, Barbados and Australia (Khan et al., 2013). Hale et al. (2017) screened 63 accessions of different *Saccharum* species at the USDA-ARS-Sugarcane Research Unit (SRU) in Houma, LA, for survival under artificially-induced freezing and found several genotypes, especially *S. spontaneum*, that are cold tolerant. Their heritability estimate also shows that about half of the freezing response is under genetic control. Burner et al. (2017) reported that early generation hybrids of sugarcane × *S. spontaneum* had more cold tolerance than commercial varieties (Tai & Miller, 1996a).

***Erianthus* spp.**

Erianthus is similar to sugarcane in appearance and phenology and it is adapted to India, China, Southeastern Asia and the Mediterranean (Cai et al., 2005; Piperidis et al., 2000) and yields almost equal dry biomass (48.8 Mg ha^{-1} in Florida) as sugarcane (Mislevy et al., 1989; Stricker et al.,

1993). The Asian species of *Erianthus* have been of particular interest in evolution of sugarcane (Jackson & Henry, 2011). *E. arundinaceus* (Rez.) Jeswit shows high biomass production, good ratooning and great biotic and abiotic stress tolerance ability (Jackson & Henry, 2011). Its cross-compatibility with sugarcane makes it useful in improving sugarcane for production (D'Hont et al., 1995; Matsunami et al., 2018). Using *Erianthus* for intergeneric hybridization has resulted in introgression of several favorable traits, such as, high polyphenol content in roots, nematode resistance and resistance to drought (Fukuhara et al., 2013; Bhuiyan et al., 2016; Nair et al., 2017). Pachakkil et al. (2019) has recently reported a significant positive correlation between number of *E. arundinaceus* chromosomes in *Saccharum* × *Erianthus* intergeneric hybrids, and their dry matter yield, stalk weight and stalk diameter. Some *E. arundinaceus* accessions and hybrids of sugarcane × *Erianthus* had previously been studied for cold tolerance in Arkansas, USA (Burner and Hale, unpublished data; Burner et al., 2017; Hale et al., 2017) that show an improved capacity of cold tolerance among these accessions. Some accessions of *E. arundinaceous* had shown overwintering ability in temperate zones of northern Japan (Ando et al., 2011). Taxa found in subtropical India (29 ºN) had been seen to have survived at a minimum temperature of 6-9 ºC (Ram et al., 2001). Ram et al. (2001) had identified hybrids between *Erianthus* and *Saccharum* having cold tolerance, hence it can be a potential source of cold tolerant genes. However, the large genetic distance between *Erianthus* and *Saccharum* results in cross-incompatibility which is a major limitation in producing intergeneric hybrids (Sobral et al., 1994; Alix et al., 1998; Cai et al., 2005; Pachakkil et al., 2019). Further limitations of using *Erianthus* for breeding arises while distinguishing true intergeneric hybrids from self-progeny (Jackson & Henry, 2011).

***Miscanthus* spp.**

Miscanthus is a perennial C_4 grass, native to east Asia (Clifton-Brown et al., 2008) with a close homology to sugarcane (Sobral et al., 1994; Amalraj & Balasundaram, 2006; Sacks et al., 2013). The two genera were estimated to have separated from a common ancestor about 3.64 mya (Tsuruta et al., 2017). *Miscanthus* spp., a member of so-called "*Saccharum* complex" (Mukherjee, 1957), is cold tolerant and can tolerate freezing temperatures (Clifton-Brown and Lewandowski, 2000). In contrast to *S. spontaneum*, the natural range of *Miscanthus* extends much further north, to ~50 °N in eastern Russia and to environments as cold as USDA hardiness zone 3 (average annual minimum temperature of -34.4 to -40.0 °C) (Clifton-Brown et al., 2015; Clark et al., 2018). *Miscanthus* ×*giganteus*, a sterile, triploid between tetraploid *M. sacchariflorus* (Maxim.) Hack. and diploid *M. sinensis* Andersson, is currently a promising biomass crop in northern latitudes of the world, largely due to its cold tolerance nature (Naidu et al., 2003; Clifton-Brown, Stampfl, & Jones, 2004; Heaton et al., 2004; 2008). This hybrid yields 59% greater biomass in the US Midwest compared to Maize (Dohleman & Long, 2009). In southern England low temperature limits production of Maize, whereas *Miscanthus* ×*giganteus* yields close to 30 t/ha (Beale & Long, 1995). Głowacka et al., (2014) reported when *M. sacchariflorus* accessions are treated with 10°C of chilling for 11 days, some of them show a better photosynthetic rate than that of *Miscanthus* ×*giganteus*. These accessions could survive night-time frost and photosynthesize at >40% better than *Miscanthus* ×*giganteus* even after being grown at 15°C (Głowacka et al., 2015). *M. sacchariflorus* accessions could be found in Siberia in eastern Russia (~50°N), which marks the northern limit of adaptation range of this plant (Clark et al., 2016). This exceptional ability of *Miscanthus* for greater cold-tolerance, adaptation to high latitude environments including temperate and sub-arctic environments, higher biomass production capacity, and biotic and abiotic

stress resistance compared to *S. spontaneum*, makes *Miscanthus* a better source of genes for improving sugarcane (Chen & Lo, 1989; Miller et al., 2005).

Saccharum × *Miscanthus* intergeneric hybrids (miscane)

Hybridization of *Miscanthus* with sugarcane had been shown to produce viable offspring (Lam et al., 2009). Research done in Taiwan and the U.S. during 1980s and 1990s proved that introgression of traits from *Miscanthus* into sugarcane through backcrossing can be a viable option (Chen & Lo, 1989; Tai et al., 1991). Originally the cross breeding was done to introgress disease-resistance genes from *Miscanthus* into sugarcane, however increase in biomass production was also observed in these hybrid genotypes which indicated a promise of using them as biomass energy producers (Sacks et al., 2013; Chen & Danao, 2015). These hybrids of *Saccharum* × *Miscanthus*, often termed as 'miscanes' (Park et al., 2011) have been reported to occur naturally (Price & Daniels, 1968; Grivet et al., 2006). However, artificial crossings were recently done between sugarcane and *Miscanthus* to generate self-compatible intergeneric hybrids (Dong, 2017; Sharma, unpublished data). Burner et al. (2017) reported ratoon cold tolerance and overwintering ability in miscane genotypes derived from *M. sinensis* Andersson at a minimum air temperature of -17.3 ºC in Arkansas, USA. Glowacka et al. (2016) confirmed that the chilling tolerance of *Miscanthus* can be transferred to sugarcane by hybridization of the two species. Hence cold tolerance genes transferred from *Miscanthus* to sugarcane, is a promising aspect in achieving high biomass production under temperate conditions (Lam et al., 2009).

In summary, the high biomass production capacity of sugarcane is largely limited in tropics due to its physiological susceptibility to low temperature. There had been attempts to improve cold-tolerance of sugarcane through breeding. *Miscanthus* as a source of genes for improving sugarcane for cold-tolerance through intergeneric hybridization has been identified. However,

understanding the genetic variation, identification of traits for selection and the potential of these intergeneric hybrids as promising biomass energy crops, is largely limited. A study of these aspects would provide a crucial knowledge base for improving lignocellulosic biomass production in high latitudes.

Objectives and composition of this thesis

This study was conducted to evaluate the performance of *Saccharum* × *Miscanthus* intergeneric hybrids (miscane) under chilling stress and their capacity as potential biomass energy producers in cooler regions of the world. Observation of photosynthetic traits such as CO_2 assimilation rate and chlorophyll fluorescence are important to elucidate chilling tolerance capacity of a plant. This study focuses on the response of genotypes to a varying degree of chilling stresses to identify best performing genotypes and key traits for selection. This dissertation consists of 5 chapters. Chapter 2 deals with the genotypic variability of photosynthetic traits in miscane genotypes in response to long-term chilling stress. Screening of miscane genotypes was performed in this experiment in a greenhouse based on photosynthetic gas exchange rate (A_n) and maximum fluorescence of dark-adapted leaves (F_v/F_m). Genotypes were grouped based on the range of responses observed in this experiment. Chapter 3 examines the chilling tolerance photosynthesis of miscane genotypes, selected based on their performance in the initial screening experiment, under varying degrees and duration of chilling stress and their subsequent recovery when the temperature was changed back to normal using growth chambers. Chapter 4 evaluates the performance of miscane genotypes under warm-temperate field conditions in Sapporo, Japan. This study observes the seasonal variation in photosynthesis and records key morphological and biomass traits. Broad-sense heritabilities (H_B^2) were determined for seasonal photosynthesis, morphological and biomass traits in order to identify key traits for selection. Chapter 5 links the overall study and discusses the key

findings in the light of previously published literature. Limitations of the current study and future avenues of research were also discussed in this chapter.

Chapter 2: Screening of *Saccharum* × *Miscanthus* intergeneric hybrids for chilling tolerance

Introduction

Chilling (0-12 °C) is an important climatic factor that can adversely impact plant growth, particularly at higher latitudes (Powles et al., 1983; Bongi & Long, 1987). Untimely chilling, especially in the early stages of plant growth, may cause irreversible damage to the plant's photosynthetic system, resulting in severe decreases in seedling establishment and survival. Although the effects of chilling depend on its intensity, duration, the growth stage of the plant, and the associated environment of the plant, chilling is one of the leading challenges for establishment of warm-season annual crops in temperate zones (Long, 1999; Long & Spence, 2013; Sage et al., 2015; Głowacka et al., 2014; Friesen & Sage, 2016). Additionally, success of perennial crops in temperate environments depends on their survival and vigor when exposed to low temperatures that are not optimally conducive to growth. In particular, having photosynthetically active leaves early and late in the season, when chilling temperatures are common at temperate latitudes, helps perennial crops take maximal advantage of the potential growing season and of available solar radiation, thereby facilitating high biomass yields (Dohleman & Long, 2009; Friesen et al., 2014; Głowacka et al., 2014; Głowacka et al., 2015).

Sugarcane is one of humanity's most important and productive crops, but it is a tropical-adapted, warm-season, C_4, perennial grass that is especially vulnerable to chilling injury. Sugarcane currently produces more biomass worldwide than any other crop, with total production of nearly 1.9 billion Mg yr^{-1}, on 26.7 Mha (FAOSTAT, 2016), and a peak dry matter yield >100 dry Mg ha^{-1} yr^{-1} (Waclawovsky et al., 2010). In addition to production of purified sugar for human consumption, sugarcane can be used as a lignocellulosic biomass or sugar feedstock for bioethanol production (Santiago et al., 2010; Ge et al., 2011). Currently, 102.4 billion liters of fuel ethanol is

produced worldwide (RFA, 2017), with US leading the production with 58 billion liters of ethanol (AMIS, 2017) mainly produced from maize, whereas Brazil leads in sugarcane ethanol production with 28 billion liters (MAPA, 2018). If used as a dedicated energy crop, sugarcane is sometimes referred to as energycane (Matsuoka et al., 2014).

Modern sugarcane cultivars are interspecific hybrids consisting primarily of *Saccharum officinarum* L., with an additional minority percentage of genes introgressed from *S. spontaneum* L. (typically for resistance to abiotic and biotic stresses) in a process historically referred to as nobilization (Stevenson, 1965; Sreenivasan & Ahloowalia, 1987; Roach, 1989; D'Hont et al., 1996; Fageria et al., 2013; Li et al., 2017). Other *Saccharum* species, such as *S. robustum* Brandes & Jeswiet ex Grassl, *S. barberi* Jeswiet, and *S. sinense* Roxb. amend. Jeswiet, may also have contributed genes to modern sugarcane cultivars but the extent of these contributions is less than that of *S. officinarum* and *S. spontaneum* (D'Hont et al., 1996; Hoarau et al., 2001; Piperidis et al., 2010; Andru et al., 2011).

Though interspecific hybridization has emerged as an efficient tool for improving sugarcane (Fageria et al., 2013), insufficient adaptation under temperate conditions, especially temperatures <18 °C has been a persistent problem (Du et al., 1999a, b; Sage et al., 2013), especially at the highest altitude and latitude extremes of its commercial production (e.g. FL and LA, USA). Głowacka et al. (2015) described sugarcane as one of the most chilling-sensitive crops in the world (Grantz, 1989). At temperatures below 20 °C, sugarcane leaf production slows, and below 10 to 15 °C growth ceases completely (Allison et al., 2007). Photosynthesis in sugarcane ceases between 8 to 12 °C (Nose et al., 1994; Fageria et al., 2013) and severe frost (-5 to -7 °C) can completely kill the aboveground plant (Sloan & Farquhar, 1978). *S. spontaneum* has been used as a source of genes for improving tolerance to low temperatures in commercial sugarcane (Moore,

1987; Fageria et al., 2013; Jackson, 2013; Khan et al., 2013), but progress has been limited because the donor species is not typically adapted to cold temperate environments (Hale et al., 2013; Knoll et al., 2013; Friesen et al., 2014).

In contrast to *S. spontaneum*, the natural range of *Miscanthus* extends much further north, to ~50 °N in eastern Russia and to environments as cold as USDA hardiness zone 3 (average annual minimum temperature of -34.4 to -40.0 °C) (Clifton-Brown et al., 2015; Clark et al., 2018). Although the majority of C_4 species are of tropical origin and adapted to warm environments, the genus *Miscanthus* is among the few exceptions that are adapted to cold-temperate environments (Heaton et al., 2010; Jones, 2011; Long & Spence, 2013; Jiao et al., 2017). In particular, *Miscanthus*, has a high degree of chilling tolerance, including exceptional photosynthetic capacity at low temperatures, compared to other warm-season C_4 perennial grasses, such as sugarcane (Beale et al., 1996; Long & Spence, 2013; Friesen et al., 2014; Fonteyne et al., 2016; Głowacka et al., 2015). In addition, *Miscanthus* rhizomes can tolerate freezing while dormant over the winter (Clifton-Brown & Lewandowski, 2000), and also show quick recovery of aboveground organs after chilling, which are both necessary for producing high biomass in cold temperate environments (Friesen et al., 2014; Głowacka et al., 2014). Thus, *Miscanthus* is considered one of the most suitable perennial grasses for biomass production in temperate environments (Iris Lewandowski, 2013), which can be attributed largely to its chilling tolerant C_4 photosynthesis.

The C_4 photosynthesis of *Miscanthus* is more efficient than C_3 species under warm temperatures (Beale & Long, 1995, 1997; Beale et al., 1999), yet more productive than most C_4 species under chilling temperatures (Dohleman & Long, 2009). Because *Miscanthus* can maintain a high photosynthetic CO_2 assimilation rate under chilling temperatures, it can produce an active canopy early in spring and late in autumn, giving it the benefit of capturing solar radiation over a

long growing season (Beale & Long, 1995; Beale et al., 1996; Dohleman & Long, 2009; Dohleman et al., 2009). For example, a *Miscanthus* hybrid evaluated in Germany was observed to produce shoots at a temperature as low as 6 °C (Farrell et al., 2006) and survived after prolonged exposure to temperatures <-6.5 °C (Clifton-Brown & Lewandowski, 2000; Farrell et al., 2006). Thus, we expect that *Miscanthus* has the potential to be a superior source of genes to *S. spontaneum*, for improving chilling tolerance of commercial sugarcane and energycane.

Molecular genetics studies indicate that *Miscanthus* and *Saccharum* are closely related (Sobral et al., 1994; Amalraj & Balasundaram, 2006; Sacks et al., 2013). The two genera were estimated to have separated from a common ancestor about 3.64 mya (Tsuruta et al., 2017). Moreover, intergeneric hybrids of *Saccharum* and *Miscanthus* have been bred previously, and these intergeneric hybrids are often termed miscanes. Miscanes have been studied since the late 1940s for their biomass production and adaptive traits (Li, 1948, 1961; Loh & Wu, 1949; Price, 1965; Chen & Lo, 1989; Xiao & Tai, 1994; Burner, 1997; Chen et al., 2000; Fageria et al., 2013; Głowacka et al., 2015). Miscanes show promise as a potential cellulosic biomass crop, given that they typically have strong, thick culms, long stem and high biomass-yield potential (Burner et al., 2015; Kar et al., unpublished data). Burner et al. (2009) reported that one miscane genotype studied in Arkansas, USA produced more biomass than *M.* ×*giganteus* Greef & Deuter ex Hodkinson & Renvoize, *M. sinensis* Andersson or the switchgrass (*Panicum virgatum* L.) 'Alamo'. Moreover, Burner et al. (2009) reported that miscanes overwintered in Boonville, Arkansas, USA, where they were subjected to a minimum winter air temperature of -14 °C. Sacks et al. 2013 suggested that miscanes could also be a potential biomass crop especially under warm-temperate or subtropical regions, through combination of key traits from its parents, including high biomass and late flowering capacity from sugarcane and high culm density, low sugar, chilling tolerance and dry

down traits from *Miscanthus*. Thus, by incorporating high biomass traits from sugarcane and cold-tolerance traits from *Miscanthus*, we expect that miscanes have potential to become a valuable lignocellulosic biomass feedstock crop in warm temperate environments and a source of genes to confer chilling tolerance in sugarcane.

Little information is currently available on the photosynthetic response of miscanes to chilling temperatures. Though Głowacka et al. (2016) reported a promising chilling-response for miscanes, they studied only three individuals. Moreover, the individuals studied previously were from crosses made in the 1980s, and thus, Głowacka et al. (2016) were able to compare the miscanes to only one of the three sugarcane parents and to none of the *Miscanthus* parents, which were of unknown provenance (even the species of the *Miscanthus* parent was unknown for two of the three progeny). Given that *Miscanthus* and sugarcane may perform very differently under chilling temperatures and that there may be variation for chilling tolerance within each genus, there is a need to evaluate a larger set of miscane progeny to determine what is typical and what range of variation might be expected. Moreover, there is a need to compare the photosynthetic response of miscanes with their respective parents to obtain an initial understanding of the trait's inheritance. To address these gaps in knowledge, the present study evaluated photosynthetic response to chilling of 18 miscane genotypes and their respective parental genotypes, including two species of *Miscanthus*, *M. sacchariflorus* and *M. sinensis*.

Materials and Methods

Plant materials

Two sugarcane parents ('KR 05-619', and 'KY 06-139'), two *Miscanthus* parents (*M. sacchariflorus* 'Miyakonojo', and *M. sinensis* 'Shiozuka'), and 18 miscane F_1 progeny were studied (Table 2-1). The sugarcane parents were breeding lines developed in the Sugarcane

Breeding Station, National Agriculture and Food Research Organization, Tanegashima, Japan (31° 44′ N, 131° 4′ E). The *Miscanthus* parents were selections from Hokkaido University. *M. sinensis* 'Shiozuka' was collected from Tokushima Prefecture, Japan (36° N, 138° E) and it is well adapted to Hokkaido (43° 04′ N, 141° 20′ E) conditions (data not shown). *M. sacchariflorus* 'Miyakonojo' was collected from Miyazaki Prefecture, Japan (31° 43′ N, 131° 4′ E) and it has survived over multiple winters in Hokkaido (data not shown). The miscanes were bred by Mr. Yoshifumi Terajima at the Tropical Agricultural Research Front of the Japan International Research Center for Agricultural Sciences in Ishigaki, Okinawa, Japan. Two of the miscanes were derived from *M. sinensis*, and the remaining 16 were derived from *M. sacchariflorus*.

Ramets of each genotype were obtained for replicated experiments by vegetatively propagating from belowground stems. For *Miscanthus*, rhizome pieces were cut to 5 cm length. For sugarcanes and miscanes, tillers with axillary buds were cut to 5 cm length. Stem divisions were established in plastic pots (dia. = 15 cm, h = 15 cm, vol. = 2 L) containing soilless medium consisting of compost, vermiculite, calcined clay, and peat moss (Forex Mori Sangyo Co., Ltd., Hokkaido, Japan). At least three rhizome pieces or tillers of a single genotype were planted in each pot. Stem divisions were planted on Oct 28, 2016. At planting and again at the start of each experiment, 15 g of 12-9-12 slow-release fertilizer (Kumiai Grassland No. 8, Hokkaido Fertilizer Co., Ltd., Japan) was added to each pot. Plants were established in a greenhouse at Hokkaido University in Sapporo, Japan (43.07° N, 141.33° E), with temperatures maintained at 22-25/13-15 °C day/night, with natural photoperiod. Irrigation was provided each day as needed.

Greenhouse experiment on long-duration chilling stress

The greenhouse experiment was a randomized complete block design, with pots randomly arranged within each of three blocks. Each block included one pot of each of the 22 entries (Table

2-1). In order to limit edge effects, pots were rearranged randomly within each block, each day of the experiment.

From the start of the experiment, on 9 December 2016, 6 weeks old plants were given 21 additional days of warm conditions (22-25 °C during the day, from 6:00 am to 8:00 pm, and 13-15 °C during the night) with 14 hour photoperiod (6:00 am to 8:00 pm day). After the initial warm establishment, plants were subsequently challenged for 14 days with chilling temperatures (12-13 °C during the day, from 8:00 am to 6:00 pm, and 7-9 °C during the night). Considering the overcast nature of the sky along with short day length commonly observed during the months of Dec.-Jan. in Hokkaido, fluorescent lights (32-W, white; FHF 32EX-N-HX, NEC Lighting Ltd., Tokyo, Japan) supplemented sunlight to maintain the photoperiod. Fluorescent lights provided 100 µmol m^{-2} s^{-1} of PPFD at canopy level. As the saturation light intensity for photosynthesis is determined by the light intensity at which the plants were grown, following Singh et al., (1974) and Usuda et al., (1985), during the warm establishment period, initial pre-chilling measurements of net photosynthetic CO_2 assimilation rates at a photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1} (A_{1000}) and a CO_2 concentration set to 400 µmol mol^{-1} were taken on all of the plants (pots) between 10:00 am to 2:00 pm over a two-day period on days 20 and 21. Measurements of A_{1000} were also taken on each plant on days 7 and 14 of the chilling period. On each night following A_{1000} measurements, maximum quantum yield of photosystem II (F_v/F_m) in dark-adapted leaves was measured on each plant between 0:00 to 2:00 am. Evaluations after seven and 14 days of chilling treatment enabled us to mimic lengthy cold waves that occur during the winter in sub-tropical production environments.

All plant measurements were taken on the youngest fully expanded leaves of each pot. For A_{1000} measurements, an individual leaf was enclosed in a controlled-environment cuvette of a

steady-state photosynthesis system (LI6400XT, LI-COR Bioscience, Lincoln, NE, USA). F_v/F_m was measured with a chlorophyll fluorometer (Junior-PAM CFMG0700B, Heinz Walz GmbH, Effeltrich, Germany). Temperature at canopy height was recorded at 10 min. intervals for the duration of the experiment with a data-logger (Thermo Recorder TR 72U, T&D Corporation, Matsumoto, Japan) and is shown in Fig. 2-S1. Irradiance inside the greenhouse was recorded with a quantum sensor at 10 min. interval (MIJ-14PARII, Environmental Measurement Japan CO. LTD., Fukuoka, Japan) and is shown in Fig. 2-S2.

Data analysis

Statistical analyses for both experiments were performed with SAS procedure GLIMMIX (version 9.4, SAS Institute Inc., Cary, NC, USA). Data from each of the measured response variables were analyzed by fitting a repeated measures mixed model, with a covariance structure for the repeated measurements selected using the Akaike information criterion corrected (AICc) (Hurvich & Tsai, 1989), from several alternative candidate models. In case of the greenhouse experiment, the AICc selected a heterogeneous compound symmetry covariance structure for the photosynthesis response data and a first-order autoregressive covariance structure for the fluorescence (F_v/F_m) data.

For the miscane F_1 full-sib family, sugarcane 'KR 05-619' × *Miscanthus sacchariflorus* 'Miyakonojo' (n = 13), and miscane F_1 half-sib family, sugarcane 'KR 05-619' or 'KY 06-139 × *Miscanthus sacchariflorus* 'Miyakonojo' (n = 16), completely random model analyses of variance were also conducted using restricted maximum likelihood via SAS procedure MIXED (version 9.4, SAS Institute Inc., Cary, NC, USA) to test the effects genotype and block in the greenhouse experiment:

$$Y = Genotype + Block + Error.$$

where the response variable Y was A_{1000} or F_v/F_m. Variance components were estimated for each of the sources of variation in the model. Broad-sense heritability (H^2) on an individual plant basis was calculated using the variance components based on following the equation (Gusmini & Wehner, 2004; Tena et al., 2016):

$$H^2 = \frac{\sigma_G^2}{\sigma_P^2} = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_E^2}$$

where σ_G^2 is the total genetic variance, σ_P^2 is the total phenotypic variance and σ_E^2 is the environmental variance. In this case, σ_G^2 represents the variation among miscane progeny genotypes, and σ_E^2 represents the interaction between genotype and block.

The Shapiro-Wilk's test statistic for normality (W) was calculated using R v 3.5.1 (R Core Team, 2015), in addition, Pearson's coefficient of skewness (y_1) and coefficient of kurtosis (y_2) for net CO_2 assimilation rate at 1000 µmols m^{-2} s^{-1} photosynthetic photon flux density (A_{1000}) and maximum quantum yield of photosystem II (F_v/F_m) of dark adapted leaves on the 14th day of chilling treatment for both full sib and half sib F_1s were calculated using "e1071" package in R v 3.5.1 (R Core Team, 2015) to detect additive effects of alleles from parental genotypes and presence of dominance genetic variation.

Results

Genotypic variation among entries under initial warm conditions (22-25 °C/13-15 °C day/night) in a greenhouse

In the greenhouse experiment, at the end of the warm establishment period, rates of net photosynthetic CO_2 assimilation were high for all entries, as expected; however, significant initial differences among entries were observed (Table 2-2). Similarly, initial values for F_v/F_m were high (0.794-0.823) but there were no significant differences among the 22 entries. For the sugarcane

parents, initial rates of A_{1000} at warm temperatures (29.2-29.9 μmol m^{-2} s^{-1}) were significantly greater than those for the *Miscanthus* parents (19.7-21.8 μmol m^{-2} s^{-1}). For the miscanes, initial rates of A_{1000} at warm temperatures ranged from 18.1-29.6 μmol m^{-2} s^{-1}, and none were significantly higher than the sugarcanes or lower than the *Miscanthus* parents. Five of the miscanes ('JM 14-47', 'JM 14-59', 'JM 14-60', 'JM 14-72', and 'JM 14-88') had initial rates of A_{1000} that were not significantly different from their sugarcane parent but significantly higher than their *Miscanthus* parent.

Response to seven days of chilling (12-13 °C/7-9 °C day/night) in a greenhouse

After seven days of chilling treatment in the greenhouse, A_{1000} of the *Miscanthus* parents (13.4-15.8 μmol m^{-2} s^{-1}) were significantly higher than those of the sugarcane parents (7.9-8.1 μmol m^{-2} s^{-1}), though all parents had significantly lower CO_2 assimilation rates than during the pre-chilling warm treatment (Table 2-2). However, the *Miscanthus* parents retained substantially more of their pre-chilling photosynthetic CO_2 assimilation rates after seven days of chilling (68-72%) than the sugarcanes (27%). Seven of the 18 miscane genotypes ('JM 14-09', 'JM 14-49', 'JM 14-52', 'JM 14-55', 'JM 14-57', 'JM 14-59', and 'JM 14-60') exhibited significantly higher CO_2 assimilation rates than their chilling-sensitive sugarcane parents, and were not significantly different from their chilling-tolerant *Miscanthus* parents, after seven days of chilling. These seven best-performing miscanes retained 48-77% of their pre-chilling photosynthetic CO_2 assimilation rates after seven days of chilling.

F_v/F_m of the *Miscanthus* parents after seven days of chilling (0.803-0.808) were not significantly different from their values during the warm conditions (Table 2-2). In contrast, F_v/F_m of the sugarcane parents after seven days of chilling (0.748-0.750) were significantly lower than those of the *Miscanthus* parents and lower than the pre-chilling values of the sugarcane parents.

Two miscane genotypes ('JM 14-06' and 'JM 14-09') had F_v/F_m after seven days of chilling that were not significantly different from their chilling-tolerant *M. sinensis* parent, and eight miscanes had values intermediate to and significantly different from both their sugarcane and *Miscanthus* parents ('JM 14-50', 'JM 14-51', 'JM 14-52', 'JM 14-55', 'JM 14-57', 'JM 14-60', 'JM 14-61', and 'JM 14-72').

Response to 14 days of chilling (12-13 °C/7-9 °C day/night) in a greenhouse

After two weeks of chilling treatment inside the greenhouse, the differences between the *Miscanthus* and sugarcane parents were greater than after only seven days of chilling (Table 2-2). A_{1000} of the *Miscanthus* parents (12.7-14.4 μmol m^{-2} s^{-1}) were more than double, and significantly higher, than the sugarcane parents (5.6-6.1 μmol m^{-2} s^{-1}), after 14 days of chilling treatment, though the reductions in CO_2 assimilation from seven to 14 days of chilling were not significant for each of the parents. The *Miscanthus* parents continued to retain more of their pre-chilling photosynthetic CO_2 assimilation rates (64-66%) than the sugarcane parents (19-20%). Of the 18 miscane genotypes, only four ('JM 14-09', 'JM 14-49', 'JM 14-55', and 'JM 14-72') had significantly higher CO_2 assimilation rates than their chilling-sensitive sugarcane parents, but notably two of these ('JM 14-09' and 'JM 14-55') did not differ from their highly tolerant *Miscanthus* parents, after 14 days of chilling. After 14 days of chilling, 'JM 14-09' retained 49% of its pre-chilling photosynthetic CO_2 assimilation rate and 'JM 14-55' retained 72%. Also notable was that 'JM 14-72' was the only miscane genotype that had rates of CO_2 assimilation that were high and not significantly different from the sugarcanes under the initial warm conditions, yet remained among the top performers after 14 days of chilling. Miscane genotypes 'JM 14-50', 'JM 14-51', 'JM 14-57', 'JM 14-60', and 'JM 14-61' showed a significant drop, between 26% and 47%, in their photosynthetic rates compared to their CO_2 fixation levels after 7 days of cold treatment, but the

remaining genotypes did not show any significant reductions in photosynthesis during the same period (Table 2-2).

F_v/F_m of the *M. sacchariflorus* parent after 14 days of chilling (0.800 ± 0.002) was not significantly different from its values after 7 days of chilling or during the warm conditions (Table 2-2). Though the F_v/F_m after 14 days of chilling for the *M. sinensis* parent (0.797 ± 0.002) was not significantly different than after 7 days of chilling, it was significantly lower than the pre-chilling values. In contrast, F_v/F_m of the sugarcane parents after 14 days of chilling (0.725-0.726) were significantly lower than after 7 days of chilling and during the initial warm period, and significantly lower than those of the *Miscanthus* parents after 14 days of chilling. Of the 18 miscane genotypes, seven ('JM 14-06', 'JM 14-09', 'JM 14-51', 'JM 14-55', 'JM 14-57', 'JM 14-61', and 'JM 14-72') had F_v/F_m that did not differ significantly from the values of the *M. sinensis* parent after 14 days of chilling, including the two *M. sinensis* progeny, but none were as high as the *M. sacchariflorus* parent. One miscane ('JM 14-76') differed significantly from and was intermediate to both parents for F_v/F_m after 14 days of chilling. The remaining ten miscane genotypes were not significantly different from the chilling-sensitive sugarcane parents for F_v/F_m after 14 days of chilling.

Heritability estimates from a greenhouse experiment

Response of the miscane F_1 progenies to chilling temperatures varied among the 18 genotypes, with some performing as well as their chilling-tolerant *Miscanthus* parents, others performing as poorly as their chilling-sensitive sugarcane parents, and many performing intermediate to both parents (Table 2-2). Quantitative variation for a trait could be due to genotypic differences, interactions between genotypes and environment, or both. However, the estimates of broad-sense heritability for both A_{1000} and F_v/F_m on day 14 of chilling were high (≥0.93), indicating that the

observed variation in the miscane F_1 full-sib and half-sib families was primarily due to genetics and not environment (Table 2-3, Table 2-S1). Additionally, broad-sense heritability estimates for the warm period and 7th day of chilling for A_{1000} and F_v/F_m were also high (≥0.79). These estimates of broad-sense heritability represent the upper potential limit of narrow-sense heritability, and the high values obtained suggest that phenotypic selection for chilling-tolerant photosynthesis in these miscane populations should be effective and efficient. Though these estimates are based on small population sizes (n = 13 or n = 16) and should be interpreted with caution, to the best of our knowledge these are the first estimates of heritability for chilling tolerant photosynthesis in miscane populations.

Table 2-1 Eighteen miscane genotypes along with their two sugarcane parents and two *Miscanthus* parents that were tested for chilling tolerance screening in greenhouse experiment.

Genotype	Type	Female parent	Male parent
'JM 14-06'	Miscane	'KY 06-139'	'Shiozuka'
'JM 14-09'	Miscane	'KR 05-619'	'Shiozuka'
'JM 14-47'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-49'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-50'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-51'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-52'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-55'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-57'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-59'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-60'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-61'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-63'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-64'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-66'	Miscane	'KR 05-619'	'Miyakonojo'
'JM 14-72'	Miscane	'KY 06-139'	'Miyakonojo'
'JM 14-76'	Miscane	'KY 06-139'	'Miyakonojo'
'JM 14-88'	Miscane	'KY 06-139'	'Miyakonojo'
'KR 05-619'	sugarcane	-	-
'KY 06-139'	sugarcane	-	-
'Miyakonojo'	*Miscanthus sacchariflorus*	-	-
'Shiozuka'	*Miscanthus sinensis*	-	-

Table 2-2 Results of greenhouse experiment showing least square means ± standard errors of net CO_2 assimilation rate at a photosynthetic photon flux density of 1000 µmol m^{-2} s^{-1} (A_{1000}) and maximum quantum yield of photosystem II (F_v/F_m) in dark-adapted leaves of 18 miscanes, and their sugarcane and *Miscanthus* parents. Measurements were taken over a two-day period on the final days (20 and 21) of an initial three week warm establishment period (22-25 °C/13-15 °C day/night). Subsequently, plants were challenged with chilling treatment (12-13 °C/7-9 °C day/night) and measured on 7th and 14th day of chilling.

		A_{1000} (µmol m^{-2} s^{-1})			F_v/F_m		
Genotype	Type	Warm	chilling 7d	chilling 14d	Warm	chilling 7d	chilling 14d
'JM 14-06'	Miscane	20.7 ± 0.5 CD* a¶	11.4 ± 0.4 BC b	10.3 ± 0.3 BC b	0.823 ± 0.002 A a	0.789 ± 0.002 B b	0.773 ± 0.002 B c
'JM 14-09'	Miscane	24.7 ± 0.5 BC a	14.4 ± 0.4 AB b	12.1 ± 0.3 AB b	0.820 ± 0.002 AB a	0.790 ± 0.002 AB b	0.780 ± 0.002 B b
'JM 14-47'	Miscane	26.9 ± 0.5 AB a	10.7 ± 0.4 BC b	8.4 ± 0.3 C b	0.808 ± 0.002 AB a	0.749 ± 0.002 D b	0.725 ± 0.002 DE c
'JM 14-49'	Miscane	25.5 ± 0.5 BC a	12.3 ± 0.4 B b	11.2 ± 0.3 B b	0.817 ± 0.002 AB a	0.745 ± 0.002 D b	0.728 ± 0.002 DE c
'JM 14-50'	Miscane	19.6 ± 0.5 CD a	15.0 ± 0.4 ABC b	8.0 ± 0.3 CD c	0.794 ± 0.002 B a	0.773 ± 0.002 BC b	0.746 ± 0.002 CD c
'JM 14-51'	Miscane	18.1 ± 0.5 D a	11.0 ± 0.4 BC b	7.9 ± 0.3 CD c	0.802 ± 0.002 B a	0.785 ± 0.002 BC b	0.772 ± 0.002 B b
'JM 14-52'	Miscane	21.4 ± 0.5 CD a	12.6 ± 0.4 AB b	10.8 ± 0.3 BC b	0.801 ± 0.002 B a	0.781 ± 0.002 BC b	0.748 ± 0.002 CD c
'JM 14-55'	Miscane	18.7 ± 0.5 CD a	14.4 ± 0.4 AB b	13.4 ± 0.3 AB b	0.819 ± 0.002 AB a	0.780 ± 0.002 BC b	0.768 ± 0.002 BC b
'JM 14-57'	Miscane	25.4 ± 0.5 BC a	15.1 ± 0.4 AB b	8.9 ± 0.3 BC c	0.819 ± 0.002 AB a	0.785 ± 0.002 BC b	0.767 ± 0.002 BC c
'JM 14-59'	Miscane	26.3 ± 0.5 AB a	12.8 ± 0.4 AB b	10.9 ± 0.3 BC b	0.808 ± 0.002 AB a	0.738 ± 0.002 D b	0.712 ± 0.002 E c
'JM 14-60'	Miscane	28.4 ± 0.4 AB a	14.0 ± 0.5 AB b	8.6 ± 0.4 BC c	0.808 ± 0.002 AB a	0.770 ± 0.002 C b	0.740 ± 0.002 CD c
'JM 14-61'	Miscane	20.5 ± 0.5 CD a	10.8 ± 0.4 BC b	8.0 ± 0.3 CD c	0.799 ± 0.002 B a	0.780 ± 0.002 BC b	0.764 ± 0.002 BC c
'JM 14-63'	Miscane	22.0 ± 0.5 BCD a	9.4 ± 0.4 BC b	7.8 ± 0.3 CD b	0.803 ± 0.002 B a	0.747 ± 0.002 D b	0.750 ± 0.002 CD b
'JM 14-64'	Miscane	18.3 ± 0.5 D a	10.7 ± 0.4 BC b	9.1 ± 0.3 BC b	0.794 ± 0.002 B a	0.764 ± 0.002 CD b	0.749 ± 0.002 CD c
'JM 14-66'	Miscane	19.8 ± 0.5 CD a	7.4 ± 0.4 C b	6.4 ± 0.3 CD b	0.815 ± 0.002 AB a	0.750 ± 0.002 D b	0.733 ± 0.002 D c

'JM 14-72'	Miscane	29.6 ± 0.5 AB a	9.9 ± 0.4 BC b	9.0 ± 0.3 BC b	0.810 ± 0.002 AB a	0.782 ± 0.002 BC b	0.774 ± 0.002 B b
'JM 14-76'	Miscane	22.2 ± 0.4 C a	8.6 ± 0.5 C b	6.6 ± 0.4 CD b	0.805 ± 0.002 AB a	0.759 ± 0.002 CD b	0.754 ± 0.002 C b
'JM 14-88'	Miscane	25.9 ± 0.5 B a	8.6 ± 0.4 C b	7.2 ± 0.3 CD b	0.808 ± 0.002 AB a	0.756 ± 0.002 CD b	0.745 ± 0.002 CD b
'KR 05-619'	Sugarcane	29.9 ± 0.5 A a	8.1 ± 0.4 C b	6.1 ± 0.3 CD b	0.810 ± 0.002 AB a	0.748 ± 0.002 D b	0.726 ± 0.002 DE c
'KY 06-139'	Sugarcane	29.2 ± 0.5 AB a	7.9 ± 0.4 C b	5.6 ± 0.3 D b	0.807 ± 0.002 AB a	0.750 ± 0.002 D b	0.725 ± 0.002 DE c
'Miyakonojo'	*M. sacchariflorus*	21.8 ± 0.5 CD a	15.8 ± 0.4 A b	14.4 ± 0.3 A b	0.812 ± 0.002 AB a	0.808 ± 0.002 A a	0.800 ± 0.002 A a
'Shiozuka'	*M. sinensis*	19.7 ± 0.5 CD a	13.4 ± 0.4 AB b	12.7 ± 0.3 AB b	0.814 ± 0.002 AB a	0.803 ± 0.002 AB ab	0.797 ± 0.002 AB b

* Upper case letters indicate comparison among genotypes within a particular period of time (Warm, 7th day of chilling treatment or 14th day of chilling treatment)

¶ Lower case letters indicate comparison across time (i.e, comparison between warm, 7th day of chilling treatment or 14th day of chilling treatment) within each genotype.

A different letter indicates significant difference ($P < 0.0001$).

Means separation was conducted by using the adjusted P-values from the Tukey-Kramer multiple comparison ($P \leq 0.05$).

Table 2-3 Variance components estimated from restricted maximum likelihood (REML), and broad-sense heritability (H^2) estimates of net CO_2 assimilation rate at 1000 μmols m^{-2} s^{-1} photosynthetic photon flux density (A_{1000}) and maximum quantum yield of photosystem II of dark adapted leaves (F_v/F_m,) for a miscane F_1 full-sib family (sugarcane 'KR 05-619' × *Miscanthus sacchariflorus* 'Miyakonojo'). Plants were grown in a greenhouse initially at 22-25 °C/13-15 °C day/night for 21 days (warm period), then challenged with 14 days of 12-13 °C/7-9 °C day/night (chilling treatment).

	Full-sib family (n = 13)					
	A_{1000}			F_v/F_m		
Source	Warm period	7th day of chilling treatment	14th day of chilling treatment	Warm period	7th day of chilling treatment	14th day of chilling treatment
Block	0.00	0.00	0.003	0.000002	0.000000	0.000004
Genotype	12.90	5.08	3.51	0.000074	0.000291	0.000335
Error	0.69	0.74	0.21	0.000015	0.000014	0.000012
H^2	0.95	0.87	0.94	0.83	0.95	0.97

Discussion

Physiology of photosynthetic chilling sensitivity in sugarcane and tolerance in Miscanthus

As expected, the sugarcane parents were highly sensitive to chilling temperatures, with greatly reduced A_{1000} and F_v/F_m after only seven days of exposure, whereas the *Miscanthus* parents were highly tolerant, even after 14 days of exposure (Table 2-2). Prior studies have also found that chilling temperatures severely reduced sugarcane photosynthesis (Friesen et al., 2014; Głowacka et al., 2014; 2016). In contrast to sugarcane, prior studies have documented exceptional chilling tolerance of photosynthesis in *Miscanthus*, (Beale et al., 1996; Long & Spence, 2013; Friesen et al., 2014; Fonteyne et al., 2016; Głowacka et al., 2015). Thus, our results for the sugarcane and *Miscanthus* parents were consistent with these prior findings.

Photosystem II is the most sensitive component of the photosynthetic apparatus to light-induced chilling damage (Long et al., 1994). Decreases in F_v/F_m in response to chilling temperatures can indicate damage to the PSII reaction centers, resulting in increased chilling-sensitivity (Głowacka et al., 2014). Thus, the F_v/F_m data from the greenhouse experiment indicated that after seven days of chilling, the photosynthetic systems of the sugarcane parents were damaged, but the *Miscanthus* parents were undamaged, and after 14 days of chilling, the sugarcanes were further damaged, but *M. sacchariflorus* parent remained undamaged and the *M. sinensis* parent was little affected.

Inheritance of chilling tolerant photosynthesis in miscanes

Both the *Miscanthus* and sugarcane parents were highly heterozygous (non-inbred), thus segregation among the F_1 progeny could be expected. For both A_{1000} and F_v/F_m traits on day 14 of chilling, of the F_1 miscane full-sib and half-sib families show high heritability estimates which

suggests that the chilling tolerance trait conferred from *Miscanthus* into sugarcane is highly heritable and selection will be feasible based on these traits (Tables 2-3, -S1).

Segregation among F_1 progeny is typical in general for crosses of highly heterozygous parents. For example, in a study on the genetics of overwintering ability in *Miscanthus*, Dong et al. (2019) identified positive and negative QTL from both winter-hardy and non-hardy parents. In previous studies, quantitative variation among *Miscanthus* and among sugarcane genotypes had been observed for CO_2 assimilation and F_v/F_m (Du et al., 1999a; Friesen et al., 2014; Głowacka et al., 2014; 2015; 2016; Tang et al., 2015). Nogueira et al. (2003) reported more than 50 genes in sugarcane that were responsive to low temperature based on gene expression. Thus, chilling tolerant photosynthesis is likely to be complex trait in miscanes, and chilling-stability of photosystem II is undoubtedly a necessary but perhaps insufficient component of overall photosynthetic tolerance to low temperatures in these populations.

In addition to being highly heterozygous, commercial sugarcane is highly polyploid (8x or more) and also typically aneuploid, so differences in chromosome number among miscane genotypes, could also result in variable performance among the progenies. Dosage effects and interactions among alleles from both parents could affect progeny performance, especially given that the tolerant *Miscanthus* parent would be expected to contribute to the F_1 progeny fewer chromosomes than the intolerant sugarcane parent. In *Miscanthus*, ploidy differences have been observed to affect photosynthetic chilling tolerance (Głowacka et al., 2010; 2014; T. Yamada, personal communication) and other traits, even when advantageous gene combinations are present (Yu et al., 2009; Głowacka et al., 2010).

Whatever the cause(s), chilling tolerant photosynthesis in F_1 miscanes appears to be a quantitative trait, with non-simple inheritance in a highly heterozygous, polyploid and likely

aneuploid genetic background. Nevertheless, we identified highly promising selections from 18 F_1 miscanes, indicating that introgression of chilling tolerance from *Miscanthus* into sugarcane should be feasible. Though *M. sacchariflorus* is typically more cold-tolerant than *M. sinensis* (Clifton-Brown et al., 2008; Sacks et al., 2013; Głowacka et al., 2014), of the two best-performing miscanes we observed after 14 days of chilling temperatures, 'JM 14-09' and 'JM 14-55', the former was derived from *M. sinensis* and the latter from *M. sacchariflorus*, demonstrating that both species can be useful sources of genes for improving chilling tolerance in sugarcane.

In summary, to the best of our knowledge, this is the first study to compare miscane progeny with their *Miscanthus* and sugarcane parents for photosynthetic chilling-tolerance. Substantial variation was observed among the progeny for CO_2 assimilation rate under chilling temperatures but there was no evidence of transgressive segregation. Duration of chilling had a large effect on the performance of the sugarcane parents and their miscane progeny but not for the *Miscanthus* parents. From seven to 14 days of chilling, the *Miscanthus* parents were relatively stable, maintaining ~2/3^{rds} of their pre-chilling CO_2 assimilation rate. In contrast, the sugarcane parents kept only ~1/3rd of their pre-chilling CO_2 assimilation rates after four days, and this dropped to ~1/5th after 14 days. Seven of 18 miscanes maintained pre-chilling CO_2 assimilation rates that were greater than their chilling-sensitive sugarcane parent after seven days of chilling, but by 14 days, only four miscanes were superior to their sugarcane parent. Notably, two miscanes, 'JM 14-09' and 'JM 14-55', had CO_2 assimilation rates after 14 days of chilling that were not significantly different from their chilling-tolerant *Miscanthus* parent, demonstrating the value of early-generation selection and the potential to improve chilling tolerance of sugarcane via introgression. High estimates of broad-sense heritability indicated that differences among miscane genotypes for chilling-tolerant photosynthesis could be detected with little bias from genotype by

environment interactions, and suggests that phenotypic selection should be effective. With genes from *Miscanthus* conferring high levels of chilling-tolerance in F_1 miscanes, it should be feasible to use these same genes to breed sugarcane and energycane cultivars that are well-adapted to colder climates than existing cultivars of these crops are, enabling an expansion onto lands that are currently economically off-limits to commercial sugarcane production. Such an advance would be expected to have a large impact on agriculture, energy, and the bioeconomy.

Chapter 3: Chilling tolerance of *Saccharum* × *Miscanthus* intergeneric hybrids

Introduction

Although C_4 is a more efficient photosynthetic process compared to C_3 under warm conditions, in terms of its light, nitrogen and water use (Long, 1983; Long & Spence, 2013), its efficiency is largely lost under cooler temperatures and a marked reduction in biomass yield is observed (Heaton et al., 2008; Long & Spence, 2013; Sage et al., 2014). C_4 plants are vulnerable to chilling conditions and most C_4 species lack frost tolerance (Sage et al., 2011; Long & Spence, 2013). Thus chilling temperature is a limitation of exploiting C_4 plants to produce biomass under abundant land and long summer days in higher latitudes (Friesen et al., 2014).

Sugarcane (*Saccharum* spp. hybrid) is one of the most important crops in the world for refined sugar as well as bioethanol production. It produces a mammoth 1.9 billion tons of biomass globally each year (FAOSTAT, 2016). Sugarcane is the most productive bioenergy feedstock on earth (Friesen et al., 2014). Although its importance as a leading commercial crop, its production is largely limited to tropics and warm subtropics (between 30° N and 35° S) due to its physiological susceptibility to cold temperatures (Du et al, 1999a, b; Głowacka et al., 2016). Sugarcane is among the most chilling sensitive crops in the world (Głowacka et al., 2016). Growth under sub-optimal temperatures (10/5 °C), sugarcane loses >98% of its photosynthetic CO_2 assimilation rate compared to when grown under warm conditions (25/20 °C) (Głowacka et al., 2014). At temperatures of 8-12 °C photosynthesis in sugarcane stops completely (Nose et al., 1994). Studies conducted in Hawaii on effect of chilling temperatures on sugarcane revealed that minimum leaf temperatures of 14 °C during winter inhibits photosynthesis, while temperatures even 20 °C in summer season can be as cool to reduce the maximum photosynthetic capacity in sugarcane (Grantz, 1989).

This lack of chilling tolerance in sugarcane can be overcome through introgression of chilling tolerance genes from species closely related to sugarcane (Głowacka et al., 2016). Cold-adapted subtropical species of wild sugarcane (*S. spontaneum*) had been crossed with commercial sugarcane (*S. officinarum*), which generated promising cultivars that can tolerate episodic chilling under warm temperate climate (Khan et al., 2013). *Miscanthus* is another valuable genetic resource for improving sugarcane due largely to its exceptional ability to tolerate chilling temperatures (Lo et al., 1978). *Miscanthus*, unlike sugarcane, by virtue of its ability to retain photosynthetically active leaves under cool temperatures, can produce comparable biomass to C_3 plants under cooler environmental conditions (Heaton et al., 2008; Long & Spence, 2013; Sage et al., 2014). *Miscanthus* ×*giganteus*, genetically a closely homologous species to sugarcane, can maintain photosynthesis even when grown at temperatures <15 °C (Farage et al., 2006). This exceptional chilling tolerance thus ensures their growth well above tropical latitudes and even up to ~ 50° N in Siberia (Pignon et al., 2019). This exceptional ability of *Miscanthus* to tolerate chilling thus theoretically provide means to introgress chilling tolerance genes in sugarcane through hybridization (Głowacka et al., 2016).

Hybrids between *Miscanthus* and *Saccharum* are sometimes referred to as 'miscanes' (Park et al., 2011). There had previous reports on hybrids between *Miscanthus* and *Saccharum* (Li, 1948, 1961; Loh & Wu, 1949; Price, 1965; Chen & Lo, 1989; Xiao & Tai, 1994; Burner, 1997; Chen et al., 2000; Fageria et al., 2013; Głowacka et al., 2015; 2016). However, reports on evidence of chilling-tolerant photosynthesis in miscanes is still limited (Burner et al., 2009; Głowacka et al., 2016; Kar et al., 2019). Głowacka et al. (2016) reported that the photosynthetic rate and maximum operating efficiency of photosystem II of three miscanes were similar to that of *Miscanthus*

×*giganteus* and greater than three sugarcane genotypes, when tested under chilling conditions in controlled environment chambers (10/5 °C).

For the current study we conducted three separate experiments to evaluate whether (i) miscanes are more chilling tolerant under long term chilling stress compared to sugarcane; (ii) miscanes perform better under short term chilling followed by recovery compared to sugarcane; (iii) miscanes perform better under low growth temperatures compared to sugarcane.

Materials and Methods

Growth chamber experiment on long-duration chilling stress

Two genotypes of miscane ('JM 14-72' and 'JM 14-88') along with their parental genotypes sugarcane 'KY 06-139' and *Miscanthus sacchariflorus* 'Miyakonojo' were selected for this experiment. Plants were grown at 25/18°C (d/n) and subsequently challenged for 23 days at 15/10°C (d/n). Measurements for A_{1500} and F_v/F_m were taken on 14th day of growth under warm temperature (25/18°C) (Control), followed by 1st, 3rd, 7th, 15th and 23rd days of chilling treatment (15/10°C). This long duration chilling stress enabled us to mimic long-duration chilling stress, which is common during late autumn in sub-tropical and warm-temperate production environments where plants often face a prolonged period of chilling temperatures before going dormant during winter.

Growth chamber experiment on short-duration chilling stress and 7 days of post-chilling recovery

A total of seven genotypes were studied in this experiment, including the two sugarcane parents, the two *Miscanthus* parents, and three miscanes selected to represent the range of responses observed in the greenhouse experiment after 14 days of chilling ('JM 14-09', 'JM 14-72', and 'JM

14-88'). A warm establishment was provided with 26/18 °C and 14 hour photoperiod for the first 14 days of the experiment. Subsequent to the warm establishment period, plants were challenged with seven days of chilling at 12/7 °C day/night. The chilling period began on day 15 of the experiment. Evaluation after 4 days of chilling treatment enabled us to mimic short-duration chilling stress, which is common during early spring and late autumn in sub-tropical and temperate production environments. After 7 days of chilling (day 21 since the experiment began), the environment was returned to warm pre-chilling conditions of 26/18 °C.

Growth chamber experiment on different growth temperatures, short-term chilling stress and 1 day of post-chilling recovery

A total of nine genotypes were studied in this experiment, including four miscanes 'JM 14-09', 'JM 14-60', 'JM 14-72' and 'JM 14-88', two sugarcane parents, 'KR 05-619' and 'KY 06-139', two *Miscanthus* parents, and one *M* ×*giganteus* 'Illinois'. Plants were grown at warm (25/18°C) (d/n) (GT 25/18°C) and cool (15/10°C) (d/n) (GT 15/10°C) growth-temperatures for 14 days, and then the warm-grown plants were subsequently challenged at 10/5°C (d/n) (CT 10/5°C) for 3 days followed by rewarming at 25/18°C (d/n) (RT 25/18°C) for 1 day.

The growth chamber experiments were completely randomized design. Each genotype was represented by three pots containing healthy and vigorous 3-week old plants. Pots were randomly arranged on four 60 cm × 50 cm trays inside two 350-L growth chambers (BioTRON LH-350S, NK Systems, Nippon Medical & Chemical Instruments Co., Ltd. Osaka, Japan). Pots were rotated randomly inside and between the two chambers on a daily basis to minimize between-chamber and within-chamber environmental effects. The growth chambers provided 400 ± 50 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) with fluorescent lamps (Hitachi FLR40S-EX-

N/M/36-A, Hitachi, Ltd., Tokyo, Japan), as measured with a quantum sensor (MIJ-14PARII, Environmental Measurement Japan CO. LTD., Fukuoka, Japan) at the top of the plant canopy.

Usuda et al., (1985) observed that Maize (a NADP-ME type C_4 like sugarcane and *Miscanthus*) plants when grown at 400 µmol m^{-2} s^{-1} of PPFD inside growth chambers show a light saturation at 1500 µmol m^{-2} s^{-1} of measured light intensity. Hence, data on net CO_2 assimilation rate were collected at a PPFD of 1500 µmol m^{-2} s^{-1} (A_{1500}). The stomatal conductance (g_s), intercellular to ambient CO_2 content (C_i/C_a), operating quantum yield of photosystem II (ϕ_{PSII}) and maximum quantum yield of photosystem II (F_v/F_m) for each plant (pot) were also observed along with the A_{1500}. All the measurements were taken on the youngest fully expanded leaf.

Data analysis

Analyses of the data were performed with SAS procedure GLIMMIX (version 9.4, SAS Institute Inc., Cary, NC, USA). A linear mixed model with repeated measures and an unstructured covariance for the repeated measurements was fit to the data to compare treatment variation along with genotypic differences. Means comparisons were implemented through the SAS LSMEANS statement with the ADJ=TUKEY option used to obtain a Tukey-Kramer multiple comparison adjustment of *P*-values for the differences of LS-means. In all cases, significance for comparisons was tested at $P<0.05$.

Results

Growth chamber experiment for long duration chilling stress

Significant difference between genotypes were observed for photosynthesis after plants were grown under 25/18°C for fourteen days. *Miscanthus sacchariflorus* 'Miyakonojo' showed highest photosynthesis while miscane genotypes 'JM 14-72' and 'JM 14-88' showed significantly lower photosynthetic rate compared to 'Miyakonojo' at this stage (Fig. 3-1a). Significant reduction in

photosynthesis compared to warm period was observed after 1 day of chilling in miscane 'JM 14-88', sugarcane 'KY 06-139' and *M. sacchariflorus* 'Miyakonojo', whereas for miscane 'JM 14-72' the significant decrease in photosynthesis was only observed after 3 days of chilling (data not shown). After 1 day of chilling *M. sacchariflorus* 'Miyakonojo' and miscane 'JM 14-72' showed significantly higher photosynthetic rate compared to sugarcane 'KY 06-139', whereas miscane genotype 'JM 14-88' showed a significantly lower photosynthesis than its *M. sacchariflorus* 'Miyakonojo' parent and did not differ significantly from its sugarcane parent (Fig. 3-1a). After 3 days of chilling treatment, both the miscanes performed at the intermediate range compared to both of their parents while *M. sacchariflorus* 'Miyakonojo' remained significantly higher than sugarcane 'KY 06-139' (Fig. 3-1a). Similar trend was observed on 7 and 23 days of chilling however, at 15 days of chilling no significant difference was observed for photosynthesis between genotypes (Fig. 3-1a). Interestingly a slight but not significant increase in photosynthetic rate was observed for all genotypes on 7 days, compared to 3 days of chilling, followed by a slight downward trend for all genotypes on 15 and 23 days (Fig. 3-1a). Only *M. sacchariflorus* 'Miyakonojo' showed a significant drop in photosynthesis on 23 days of chilling compared to its 1 day chilling photosynthesis value (data not shown).

For maximum yield of photosystem II (F_v/F_m), significant genotypic difference was only observed at 14 and 23 days of chilling treatment where *M. sacchariflorus* 'Miyakonojo' showed significantly higher F_v/F_m compared to sugarcane and miscane 'JM 14-88' on 14 days, and to only sugarcane on 23 days (Fig. 3-1b). Although all of the genotypes showed a decreasing trend in F_v/F_m with duration of chilling, significant decrease was only observed for sugarcane 'KY 06-139' on 15 and 23 days of chilling compared to its respective warm period and 1 day of chilling values (data not shown).

Growth chamber experiment on short-duration chilling stress and 7 days of post-chilling recovery

At the start of the experiment in the growth chamber, under warm temperatures, values of A_{1500}, F_v/F_m and C_i/C_a, indicated that all plants were healthy and photosynthetically active, as expected; however, significant initial differences among entries were observed (Fig. 3-2). For the sugarcane parents, initial rates of A_{1500} (28.3-29.3 μmol m^{-2} s^{-1}) at warm temperatures were high and similar to the warm period rates observed in the greenhouse experiment. Similarly, initial rates of A_{1500} for the *M. sacchariflorus* parent (29.9 ± 0.4 μmol m^{-2} s^{-1}) and miscane 'JM 14-09' (28.8 ± 0.4 μmol m^{-2} s^{-1}) were not significantly different from the sugarcane parents. However, initial A_{1500} for the *M. sinensis* parent (24.2 ± 0.4 μmol m^{-2} s^{-1}) and miscane 'JM 14-88' (23.4 ± 0.4 μmol m^{-2} s^{-1}) were significantly lower than for the sugarcane parents. Notably, miscane 'JM 14-72' had a significantly higher initial photosynthetic rate (33.2 ± 0.4 μmol m^{-2} s^{-1}) than all the other entries, which was consistent with its high initial rate in the greenhouse experiment. Initial F_v/F_m were high and similar among all entries (0.808-0.822), except for a significantly lower value for the *M. sinensis* parent (0.795 ± 0.001). No differences in initial C_i/C_a, among entries were observed.

A short four-day exposure to chilling temperatures in the growth chambers negatively impacted photosynthesis of all entries tested, but some entries were more greatly affected than others. After four days of chilling, the *Miscanthus* parents had significantly higher A_{1500} (16.0-19.7 μmol m^{-2} s^{-1}) than the sugarcane parents (9.3-9.1 μmol m^{-2} s^{-1}), and a similar significant difference was observed for F_v/F_m (0.787-0.800 for the *Miscanthus*, in contrast to 0.728-0.731 for the sugarcanes). The *M. sacchariflorus* parent had a significantly greater A_{1500} after chilling than the *M. sinensis* parent. Similarly, when exposed to four days of chilling temperatures, the *Miscanthus*

parents retained a greater percentage of their pre-chilling A_{1500} (66%) and F_v/F_m (97-99%) than the sugarcane parents (32-33% A_{1500}, and 89-90% of their pre-chilling F_v/F_m).

All three miscanes performed significantly better than the sugarcane parents for A_{1500} and F_v/F_m after four days of chilling. The miscanes were more similar to their *Misanthus* parents than their sugarcane parents, retaining between 45-60% of their pre-chilling photosynthetic rate, and 97-99% of their pre-chilling F_v/F_m. The best miscane entry, 'JM 14-09', did not significantly differ from its *M. sinensis* parent for A_{1500} and F_v/F_m after chilling. The performance of the three miscane genotypes in the growth chamber experiment in response to four days of chilling showed a similar pattern to their performance in the previous greenhouse experiment in response to seven and 14 days of chilling. In each case, 'JM 14-09' had the highest CO_2 assimilation rate, followed by 'JM 14-72', then 'JM 14-88', though there was not a significant difference between the latter two entries at each time point.

Under chilling, C_i/C_a of all entries in the growth chamber experiment increased relative to the pre-chilling values (Fig. 3-2), indicating that the intercellular CO_2 concentration increased, due to a decrease in CO_2 fixation. Means separation identified two groups of entries for C_i/C_a under chilling temperatures but there was no clear distinction between the parental species, as there had been for A_{1500} and F_v/F_m. Under chilling, miscanes 'JM 14-09', 'JM 14-72', sugarcane 'KR 05-619', and the *M. sinensis* parent had significantly higher C_i/C_a (0.701 ± 0.011, 0.769 ± 0.011, 0.709 ± 0.011 and 0.754 ± 0.011, respectively) than the other genotypes.

Seven days after post-chilling return to warm temperatures in the growth chambers two types of responses were observed: 1) full recovery, and 2) partial recovery (Fig. 3-2). Both *Miscanthus* parents and miscanes 'JM 14-09' and 'JM 14-88' fully recovered or exceeded their pre-chilling values of A_{1500} and F_v/F_m after seven days of chilling followed by seven days return

to warm temperatures. In contrast, the sugarcane parents had significantly lower A_{1500} (20.3-20.5 μmol m^{-2} s^{-1}) and F_v/F_m (0.763-0.766) at the end of the experiment than during the warm period but the end values were significantly higher than after four days of chilling, indicating a partial recovery. Thus, the sugarcane parents recovered only 69-73% of their pre-chilling photosynthetic CO_2 assimilation rates and 94% of their initial F_v/F_m by the end of the experiment, whereas recovery for the *Miscanthus* parents was 97-103% for CO_2 assimilation and 100-103% for F_v/F_m. Similarly, recovery for the miscanes 'JM 14-09' and 'JM 14-88' was 103-104% for CO_2 assimilation and 100-101% for F_v/F_m. In contrast, miscane 'JM 14-72' had partial recovery for CO_2 assimilation (77%) but a full recovery for F_v/F_m (100%). All entries fully recovered or exceeded their pre-chilling values of C_i/C_a.

Growth chamber experiment on different growth temperatures, short-term chilling stress and 1 day of post-chilling recovery

Significant genotypic difference in photosynthesis was observed after plants were grown at 25/18°C for two weeks (Fig. 3-3a). Miscane genotype 'JM 14-88' showed highest photosynthetic rate which was significantly higher than both the sugarcane genotypes, miscane 'JM 14-72', *Miscanthus sacchariflorus* 'Miyakonojo' and *M.* ×*giganteus* 'Illinois' (Fig. 3-3a). Miscane 'JM 14-09' also showed significantly high photosynthetic rate compared to both the sugarcane genotypes, miscane 'JM 14-72' and *Miscanthus sacchariflorus* 'Miyakonojo' (Fig. 3-3a). However, when the plants were grown under low-temperature (15/10°C) for two weeks, none of the genotypes, except miscane 'JM 14-60', showed significantly lower photosynthesis compared to its warm-grown photosynthesis values (Fig. 3-3a). Interestingly *M.* ×*giganteus* 'Illinois' photosynthesized at a greater rate when grown under low growth-temperature (15/10°C) compared to when grown under warm temperature (25/18°C) (Fig. 3-3a). Significantly high photosynthetic

rate, compared to sugarcane 'KR 05-619', was observed in miscane 'JM 14-09', *M.* ×*giganteus* 'Illinois', miscane 'JM 14-72' and *M. sinensis* 'Shiozuka' when plants were grown in low-temperature (Fig. 3-3a). When the warm-grown plants were chilled for 3 days at 10/5°C, miscane genotypes, 'JM 14-09' and 'JM 14-60', sugarcane 'KY 06-139' and *Miscanthus sinensis* 'Shiozuka' showed a significant reduction in photosynthesis compared to their respective pre-chilling values (Fig. 3-3a). Under chilling treatment, two miscanes, 'JM 14-72' and 'JM 14-09', showed significantly higher photosynthetic rate compared to sugarcane 'KY 06-139'. Among these two miscanes, 'JM 14-72' showed significantly higher photosynthesis compared to both the sugarcane genotypes and *Miscanthus* parental genotypes (Fig. 3-3a). Three miscanes, 'JM 14-72', 'JM 14-09' and 'JM 14-60' photosynthesized at a greater rate, although not significantly, compared to *M.* ×*giganteus* 'Illinois' (Fig. 3-3a). When photosynthetic recovery was measured on chilled plants after 1 day of rewarming, miscanes 'JM 14-09', 'JM 14-60' and 'JM 14-88' showed significantly higher photosynthesis compared to sugarcane 'KY 06-139' and *M. sinensis* 'Shiozuka' (Fig. 3-3a). Although we observed a high photosynthetic rate under chilling in miscane 'JM 14-72', during recovery it did not differ significantly from both the sugarcanes or from any of the *Miscanthus* genotypes (Fig 3-3a). All of the genotypes, except miscane 'JM 14-88' have recovered their respective pre-chilling photosynthesis values (Fig. 3-3a). We observed an increment in photosynthetic rate at recovery in all genotypes except miscanes, 'JM 14-60' and 'JM 14-72', compared to their respective pre-chilling values, however, the increment was only significant for miscane 'JM 14-88' (Fig. 3-3a). Interestingly, the photosynthetic rate of miscane 'JM 14-72' did not differ across treatments or growth-temperatures (Fig. 3-3a).

No significant difference in stomatal conductance (g_s) was observed between genotypes grown under warm growth-temperature (Fig. 3-3b). However, when plants were grown under

cooler growth-temperature (15/10°C), miscanes, 'JM 14-60', 'JM 14-72' and 'JM 14-09', sugarcane 'KY 06-139' and *Miscanthus sinensis* 'Shiozuka' showed significantly higher stomatal conductance compared to sugarcane 'KR 05-619' (Fig. 3-3b). When warm-grown plants were chilled at 10/5°C, miscanes, 'JM 14-72' and 'JM 14-60' showed a significantly higher g_s compared to *Miscanthus sacchariflorus* 'Miyakonojo' (Fig. 3-3b). After rewarming, only miscane genotype 'JM 14-60' showed a significantly higher g_s compared to all of the *Miscanthus* genotypes (Fig. 3-3b).

g_s of miscane genotypes, 'JM 14-09' and 'JM 14-60', sugarcane 'KR 05-619' and *Miscanthus sacchariflorus* 'Miyakonojo' did not differ significantly across treatments or growth temperatures (Fig. 3-3b). Lower growth-temperature and chilling both had affected in an increase in g_s compared to warm-grown values in all genotypes except for *Miscanthus sacchariflorus* 'Miyakonojo' (Fig. 3-3b) however, the increase in g_s was more affected by the former than the latter (Fig. 3-3b). Full recovery of pre-chilling g_s was achieved in all genotypes except for miscane 'JM 14-88' and sugarcane 'KY 06-139' where g_s values obtained after rewarming were significantly greater than their respective pre-chilling values (Fig. 3-3b).

Intercellular to ambient CO_2 ratio (C_i/C_a) did not differ across genotypes under different growth temperatures (Fig. 3-3c). Although, growth temperature did not result in genotypic difference, significantly higher C_i/C_a values were obtained under cooler growth temperature compared to warm growth-temperature in all the genotypes (Fig. 3-3c). Chilling (10/5°C) the warm-grown plants also resulted in a significant increase in C_i/C_a compared to pre-chilling values among all genotypes. Significant genotypic difference was only observed between 'JM 14-60' and *Miscanthus sacchariflorus* 'Miyakonojo', sugarcane 'KY 06-139', miscane 'JM 14-09', and *M.* ×*giganteus* 'Illinois' after 3 days of chilling (Fig. 3-3c). During post-chilling recovery miscane

'JM 14-60' only differed significantly from *M.* ×*giganteus* 'Illinois' (Fig. 3-3c). All genotypes had recovered their respective pre-chilling C_i/C_a after rewarming, however, the values obtained at rewarming were higher than their respective pre-chilling values (Fig. 3-3c).

Operating quantum yield of photosystem II (ϕ_{PSII}) values differed significantly across genotypes in both warm-grown and cold-grown plants (Fig. 3-3d). Miscanes 'JM 14-88' and 'JM 14-09' showed highest ϕ_{PSII} values under warm growth temperature, whereas miscane 'JM 14-60' and sugarcane 'KY 06-139' showed highest values and miscane 'JM 14-09' showed the lowest under cool growth temperature (Fig 3-3d). Lower growth temperature resulted in a lower ϕ_{PSII} among all genotypes compared to warm grown values, however, significant difference was only observed in miscanes 'JM 14-09', 'JM 14-60', 'JM 14-88', and *M.* ×*giganteus* 'Illinois' (Fig 3-3d). When warm-grown plants were chilled, significant reduction in ϕ_{PSII} was obtained in miscanes 'JM 14-09', 'JM 14-88' and both sugarcanes compared to their respective pre-chilling values (Fig. 3-3d). Significantly higher ϕ_{PSII} values were obtained post-chilling in miscanes 'JM 14-60', 'JM 14-09' and 'JM 14-72' compared to *Miscanthus sacchariflorus* 'Miyakonojo'. After rewarming however, *M.* ×*giganteus* 'Illinois', sugarcane 'KY 06-139' and all miscanes except 'JM 14-72' showed significantly higher ϕ_{PSII} values compared to sugarcane 'KR 05-619' (Fig. 3-3d). A complete recovery of pre-chilling ϕ_{PSII} were obtained by all genotypes except sugarcane 'KR 05-619', which remained significantly lower compared to its pre-chilling values (Fig 3-3d).

No significant genotypic difference was observed for maximum quantum yield of photosystem II (F_v/F_m) under both growth temperatures and rewarming (Fig. 3-3e). Although, no significant genotypic difference was found in different growth temperatures, F_v/F_m of plants grown at lower growth temperature were significantly lower in all genotypes except miscane 'JM 14-88' and all *Miscanthus* genotypes (Fig. 3-3e). Similar to photosynthesis, F_v/F_m was also recorded to

be higher when *M*. ×*giganteus* 'Illinois' plants were grown under low growth-temperatures compared to warm growth temperatures, however, the difference was not significant (Fig. 3-3e). Chilling treatment had significantly reduced F_v/F_m in all genotypes except for *Miscanthus sacchariflorus* 'Miyakonojo' (Fig. 3-3e). Significant genotypic difference in F_v/F_m was observed after chilling treatment where *Miscanthus sacchariflorus* 'Miyakonojo', and miscanes 'JM 14-60' and 'JM 14-72' showed significantly higher F_v/F_m compared to rest of the genotypes except miscane 'JM 14-09' (Fig 3-3e). All genotypes had recovered their respective pre-chilling F_v/F_m after rewarming (Fig. 3-3e).

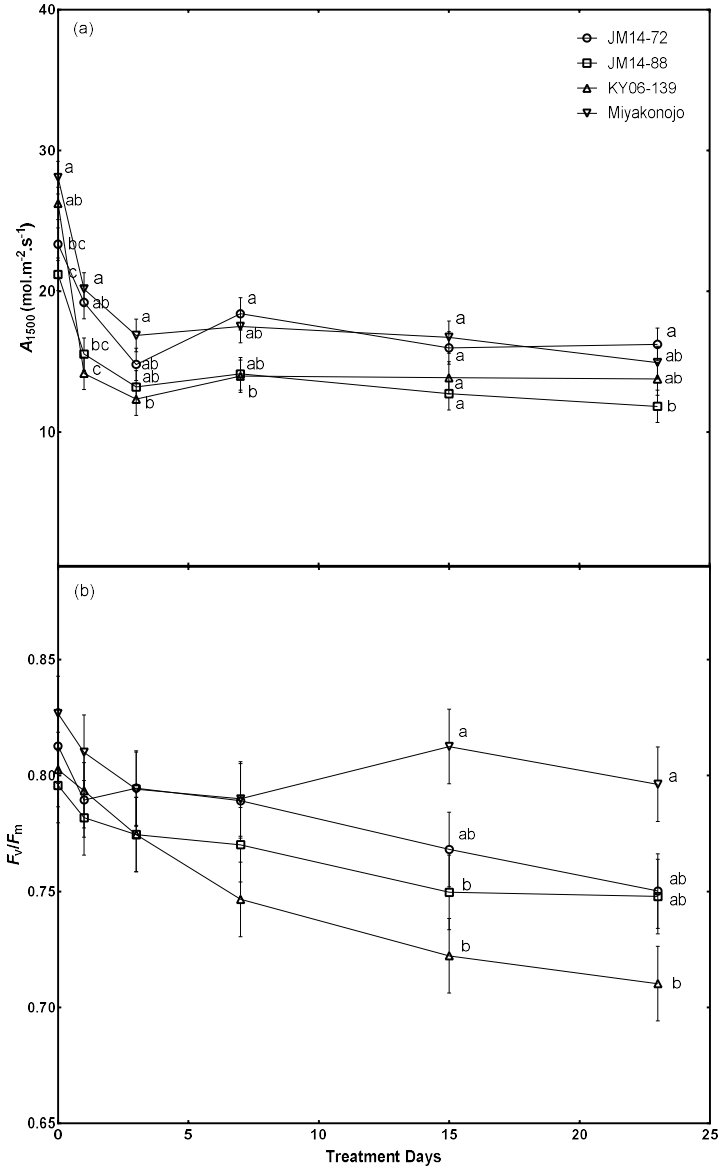

Fig. 3-1 Photosynthetic gas exchange rate (A_{1500}) (a) and maximum quantum yield of photosystem II (F_v/F_m) (b) under 23 days of chilling (15/10°C) treatment. Plants were grown at 25/18°C (d/n) and subsequently challenged for 23 days at 15/10°C (d/n). Measurements were taken on 14th day of growth under warm temperature (25/18°C) (Treatment day 0), followed by 1st, 3rd, 7th, 15th and 23rd days of chilling treatment (15/10°C). Values are least square means of Genotypes × Day (±SE). Different letters indicate significant difference (P≤0.05) between genotypes.

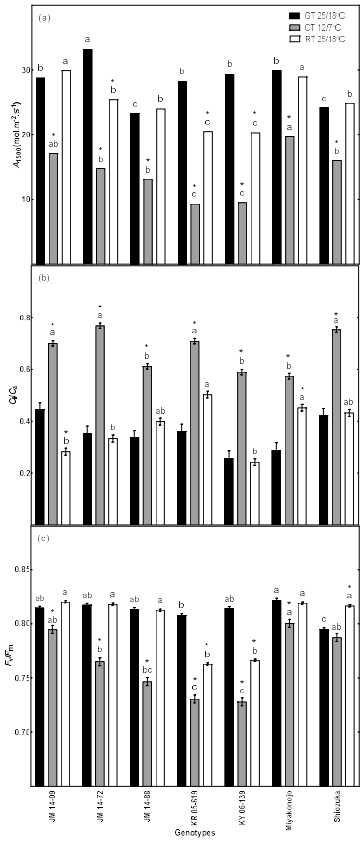

Fig. 3-2 Photosynthetic gas exchange rate (A_{1500}) (a), ratio of intercellular to ambient CO_2 partial pressure (C_i/C_a) (b), and maximum quantum yield of photosystem II (F_v/F_m) (c) of miscane, sugarcane and *Miscanthus* genotypes. Plants were grown at warm (25/18°C) (d/n) (GT 25/18°C) growth-temperatures for 14 days, and then the plants were subsequently challenged at 12/7°C (d/n) (CT 12/7°C) for four days. After seven days of chilling rewarming at 25/18°C (d/n) (RT 25/18°C) was done for 7 days. Values are Genotype × Treatment least square means (±SE). A different letter on top of a bar indicates significant difference ($P \leq 0.05$) between genotypes under each treatment level. Asterisk on top of a bar indicates a significant difference ($P \leq 0.05$) of a treatment compared to values obtained under warm growth-temperatures under each genotype.

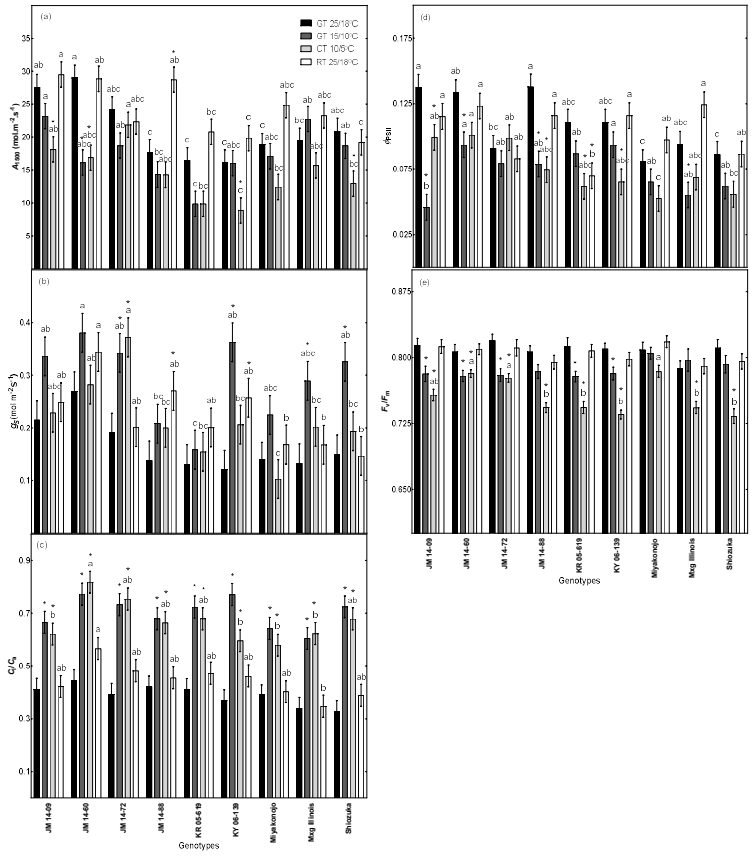

Fig. 3-3 Photosynthetic gas exchange rate (A_{1500}) (a), stomatal conductance (g_s) (b), ratio of intercellular to ambient CO_2 partial pressure (C_i/C_a) (c), operating quantum yield of photosystem II (ϕ_{PSII}) (d) and maximum quantum yield of photosystem II (F_v/F_m) (e) of miscane, sugarcane and *Miscanthus* genotypes. Plants were grown at warm (25/18°C) (d/n) (GT 25/18°C) and cool (15/10°C) (d/n) (GT 15/10°C) growth-temperatures for 14 days, and then the warm-grown plants were subsequently challenged at 10/5°C (d/n) (CT 10/5°C) for 3 days followed by rewarming at 25/18°C (d/n) (RT 25/18°C) for 1 day. Values are Genotype × Treatment least square means (±SE). A different letter on top of a bar indicates significant difference ($P \leq 0.05$) between genotypes under each treatment level. Asterisk on top of a bar indicates a significant difference ($P \leq 0.05$) of a treatment compared to values obtained under warm growth-temperatures under each genotype.

Discussion

C_4 grasses fix CO_2 through phosphoenol pyruvate carboxylase (PEPc) regeneration via pyruvate phosphate dikinase (PPDK) (Wang et al., 2008a). The product of C_4 photosynthesis is transported to bundle sheath cells in leaves where CO_2 is released through decarboxylation of this product and refixed by Rubisco (Wang et al., 2008a). This process concentrates more CO_2 compared to O_2 around rubisco, thus eliminating the deleterious photoinhibitory process (Long, 1983). This results in an increase in efficiency of light, water and nitrogen use in C_4 plants relative to C_3 plants (Long, 1983). Although this process is theoretically advantageous even under low-temperatures (Long, 1983; 1999), only a few C_4 plants are actually photosynthetically capable under cool temperatures (<15 °C) (Wang et al., 2008a). In most C_4 species low temperature can cause photoinhibition or damage to enzymes of C_4 metabolic cycle such as Pyruvate orthophosphate dikinase (PPDK) and NADP-malate dehydrogenase (NADP-MDH) (Long, 1983; Potvin et al., 1986; Du et al., 1999a; Naidu et al., 2003; Wang et al., 2008a). In addition, C_4 species are inherently Rubisco-limited under low temperature (Kubien & Sage, 2004; Sage et al., 2011). This rubisco limitation under cool condition can place a low ceiling capacity photosynthesis in C_4 plants as well as it can restrict the photochemical quenching process and predispose plants to photoinhibition (Kubien et al., 2003; Kubien & Sage, 2004a).

Although *Miscanthus* is more chilling tolerant than most C_4 species, we observed that its photosynthesis can still suffer from chilling damage, especially under severe stress. When a chilling tolerant C_4 like *M.* ×*giganteus* and a chilling sensitive C_4 like maize are grown at 25 °C are transferred to 14 °C for two days, their photosynthesis and quantum yield of photosystem II drops by 30% and 40% respectively (Wang et al., 2008a). These data are consistent with our findings. Plants with undamaged PSII reaction centers are more likely to fully recover photosynthetic CO_2 assimilation rates upon post-chilling return to warm temperatures than those

with damaged PSII, a question we investigated in growth chamber experiments 2 and 3. Photosynthetic carbon assimilation can be severely reduced under saturating light and chilling (<15 °C) temperatures, and utilization of excitation energy leads to photo-inhibition and photo-oxidation (Long, 1983; Baker et al., 1989; Long et al., 1994). CO_2 assimilation can also be reduced if stomata close in response to cold. Additionally, decreases in chlorophyll fluorescence value (F_v/F_m) are typically associated with chilling-dependent photoinhibition, which is one of the factors responsible for limiting CO_2 assimilation in mature leaves (Baker et al., 1989; Farage & Long, 1987; Farage et al., 2006; Ortiz-Lopez et al., 1990). Du et al. (1999a) found that under chilling for 3 days, sensitive sugarcane genotypes retain about 22% of their pre-chilling photosynthetic rate, while tolerant genotypes retained between 69 and 74%. Our growth-chamber experiments support these finding. This chill-induced reduction in the capacity of the leaf to assimilate CO_2 is dependent on the duration of the chill and increases with increasing photon flux density (Long et al., 1983) and its duration (Taylor & Rowley, 1971). However, in our experiment shortening the photoperiod during chilling may have induced a lesser extent of photo-inhibition and photo-oxidative damage to the tolerant genotypes.

Additionally, the substantial decrease in CO_2 assimilation we observed may also be explained by enzymatic regulation for photosynthesis under chilling. The cold labile C_4 photosynthetic enzyme pyruvate orthophosphate dikinase (PPDK) plays a limiting role under low temperature in cold-sensitive C_4 species (Hatch, 1979; Sugiyama et al., 1979; Potvin et al., 1986; Burnell, 1990; Usami et al., 1995; Wang et al., 2008a). In addition to PPDK, ribulose bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenol pyruvate carboxylase (PEPc) also limit photosynthesis under chilling (Kingston-Smith et al., 1997; Du et al., 1999a, b; Wang et al., 2008a, b). Rubisco content and activity declines proportional to the rate of decline in photosynthetic rate

(Kubien et al., 2003). Sage & McKown, (2006) proposed that in C_4 plants, Kranz anatomy limits Rubisco to bundle sheath, hence C_4s are inherently more prone to Rubisco limitation under low temperature. Du et al. (1999a) found that a sensitive sugarcane genotype, determined by a greater reduction in photosynthetic rate after chilling, showed a significant reduction in activity of all three photosynthetic enzymes, while, the tolerant genotypes showed a marked increase in activity of the enzymes especially PPDK. An increased activity of photosynthetic enzymes in tolerant genotypes thus, may have been essential in maintaining a higher rate of CO_2 assimilation during chilling in our experiments. Long et al. (1983) observed a steep increase in intercellular CO_2 content (C_i) along with photoinhibition. Thus, the increased ratio of intercellular to ambient CO_2 concentration (i.e. C_i/C_a) along with the small reduction in F_v/F_m that we observed in response to chilling in the growth chamber experiments indicated that a lesser degree of light-induced chilling damage of PSII was likely a cause of the lower rates of photosynthesis observed. This conclusion is consistent with prior studies of *Miscanthus* and sugarcane (Głowacka et al., 2014; 2016; Jiao et al., 2017).

Lower growth temperature also results in a lower rate of photosynthetic CO_2 fixation (Nie et al., 1992; Wang et al., 2008a). In our experiment we also observed a decrease in all of the genotypes except in *M.* ×*giganteus* when grown at a temperature of 15/10 °C (Fig. 3-3). This finding is consistent with prior studies (Nie et al., 1992; Naidu et al., 2004; Farage et al., 2006; Wang et al., 2008a).

During recovery from chilling after one day and seven days of warming in the growth chamber experiment (Fig. 3-2, -3), our results indicate that short-duration chilling resulted in lasting damage to PSII in the sugarcane genotypes but that the *Miscanthus* and miscanes were resilient. Głowacka et al. (2014; 2015; 2016) also found that chilling-susceptible sugarcane accessions had very low rates of recovery upon rewarming after chilling treatment, concluding that

chilling resulted in a damaged photosynthetic system for sugarcanes but not for some *Miscanthus* genotypes. Wang et al., (2008a) also observed that in *Miscanthus* ×*giganteus*, a cold tolerant biomass cultivar shows increased amount and activity of PPDK during chilling treatment which helps it maintain a stable photosynthetic rate, whereas, in maize, a chilling-sensitive species, the PPDK activity reduces with duration of chilling. In our experiemnts we also observed a higher photosynthetic rate in miscane and *Miscanthus* genotypes compared to sugarcane. This increase in the activity of photosynthetic enzymes in tolerant genotypes during chilling may have caused the observed post-chilling increment in photosynthetic rate in these genotypes over their respective pre-chilling values (Fig. 3-2, -3). In the field, resiliency to early-season and late-season temperature fluctuations is arguably more valuable than high rates of photosynthesis during chilling events per-se, as the potential for CO_2 assimilation upon return to warm temperatures is substantially greater than during short-duration chilling events.

Chapter 4: Field performance of *Saccharum* × *Miscanthus* intergeneric hybrids

Introduction

Low temperature in the early and late period of the growing season inhibits plant growth in higher latitudes, such as northern Japan (Powles et al., 1983; Bongi & Long, 1987). Chilling temperatures (0-15° C), depending on their intensity, duration, and associated environment of the plant, often cause damage to the photosynthetic apparatus in plant cells. This is especially true in chilling-sensitive plants, resulting in high mortality, poor establishment, and reduced crop vigor and productivity. Chilling temperature is one of the leading challenges for crop establishment in temperate zones (Long, 1999; Long & Spence, 2013; Głowacka et al., 2014; Sage et al., 2015; Friesen & Sage, 2016). Consequently, successful growth and development of perennial crops adapted to temperate environments depend on survival to low-temperature exposure. In addition, the capacity of plants to overwinter and maintain photosynthetically active leaves early and late in the growing season enable them to prolong their growth period, which generally leads to relatively higher yields (Dohleman & Long, 2009; Friesen et al., 2014; Głowacka et al., 2014; 2015).

Sugarcane (complex hybrids of *Saccharum officinarum* L., *Saccharum robustum* Brandes & Jeswiet ex Grassl, *Saccharum spontaneum* L., *Saccharum barberi* Jeswiet, *Saccharum sinense* Roxb. amend. Jeswiet, and possibly *Miscanthus* Anderss. spp.) (Burner et al., 2017), which is cultivated on 26.7 mha and yields nearly 1.9 billion metric tons per year (FAOSTAT, 2016) with a peak dry matter yield >100 tons (ha yr^{-1}) (Waclawovsky et al., 2010), is among the highest biomass-producing crops in the world (FAOSTAT, 2016; Głowacka et al., 2016). In addition to being the leading sugar-producing crop for human consumption, sugarcane is also used as a lignocellulosic biomass and a feedstock for bioethanol production (Santiago et al., 2010; Ge et al., 2011). Currently, worldwide production of bioethanol amounts to 102.4 billion liters (RFA, 2017),

with US leading the production at 58 billion liters (AMIS, 2017) which is mainly produced from maize. However, Brazil produces 28 billion liters of ethanol, mainly from sugarcane(MAPA, 2018).

However, the lack of environmental adaptation of sugarcane has been a persistent problem, especially in high-latitude and/or high- elevation areas, owing to its susceptibility to cold (Du et al., 1999a, b; Sage et al., 2013). Głowacka et al. (2015) described sugarcane as one of the most chilling-sensitive crop species in the world (Grantz, 1989). Its rate of leaf production decreases below 20°C and growth ceases completely below 10 to 15°C (Allison et al., 2007). Photosynthesis stops between 8 to 12 °C (Nose et al., 1994; Fageria et al., 2013) and severe frost (-5 to -7°C) can completely kill the aboveground plant (Sloan & Farquhar, 1978). Hybridization programs have been attempted to improve cold tolerance and improved adaptation of this crop to higher latitudes (Moore, 1987; Beale & Long, 1995; Lewandowski et al., 2000; Clifton-brown et al., 2001; Glowacka et al., 2016). Crosses between commercial sugarcane (hybrids of mainly *S. officinarum* and *S. spontaneum*) and *S. spontaneum* have been reported to show cold tolerance. However, these crosses are not reliable for cultivation due to their varying degree of chilling tolerance (Fageria et al., 2013; Jackson, 2013; Khan et al., 2013; Hale et al., 2013; Knoll et al., 2013; Friesen et al., 2014). In addition to *S. spontaneum*, taxa in the *Saccharum* genus can be crossed with related genera belonging to so-called "*Saccharum* complex" (Mukherjee, 1957), which includes *Miscanthus* (Chen, 1953; Chen et al., 1983), *Erianthus* (D'Hont et al., 1995; Piperidis et al., 2000; Ram et al., 2001; Pachakkil et al., 2019), *Narenga* (Mukherjee, 1957; OGTR, 2011)*, Sclerostachya* (OGTR, 2011; Moore et al., 2013), or other genetically similar C_4 perennial grasses (OGTR, 2011). Such interspecific and intergeneric hybrids often express traits, such as disease or pest resistance (Chen & Lo, 1989; Ram et al., 2001; Pachakkil et al., 2019), abiotic stress resistance (Lo et al.,

1978; Deren et al., 1991; Ram et al., 2001; Burner et al., 2009; Pachakkil et al., 2019), improved perenniality (Ando et al., 2011), and adaptation to marginal land conditions (Fageria et al., 2013; Deren et al., 1991; Chen, 1993), along with improved productivity (Ando et al., 2011; Park et al., 2011; Burner et al., 2017).

Among these related genera to *Saccharum*, *Miscanthus* is receiving a lot of focus in recent years as a potential genetic resource, especially to improve cold-tolerance in sugarcane. *Miscanthus*, a native C_4 grass of southeast Asia, unlike sugarcane, shows a high degree of cold tolerance compared to other warm-season, C_4 perennials (Stewart et al., 2009; Friesen et al., 2014; Głowacka et al., 2015; Fonteyne et al., 2016). In a study in Germany, a stay-green *Miscanthus sinensis* hybrid (Sin-H9), derived from hybridization of two *Miscanthus sinensis* populations with wide temperature ranges (Hodkinson et al., 2002), was reported to produce shoots at a temperature as low as 6 °C (Farrell et al., 2006), and survived after prolonged exposure (3 h) to temperatures <-6.5 °C (Farrell et al., 2006); (Clifton-Brown & Lewandowski, 2000). *Miscanthus* ×*giganteus* Greef and Deuter ex Hodkinson and Renvoize Aksel Olsen, a cold-tolerant, sterile, triploid hybrid between tetraploid *M. sacchariflorus* and diploid *M. sinensis*, which was collected in Yokahama, Japan in 1935, (Anderson et al., 2011) has been extensively studied since the 1980s as a potential biomass crop in Europe (Lewandowski et al., 2003) and in more recent years in the U.S. (Heaton et al., 2008; Pyter et al., 2009). The only commercial cultivar of the hybrid, *M.* ×*giganteus* 'Illinois', has shown cold tolerance and high biomass productivity over several years in high-latitude regions (Beale et al., 1996). *Miscanthus* ×*giganteus* 'Illinois' has been reported to produce 20 t of dry biomass/ha/year in Europe and North America (Price et al., 2004; Heaton et al., 2010). The high biomass-production capacity can be attributed to its C_4 photosynthetic nature, which is more resource efficient than the C_3 pathway under warm conditions (Beale & Long, 1995; 1997;

Beale et al., 1999). Close homology of *Miscanthus* to sugarcane (Amalraj & Balasundaram, 2006; Sacks et al., 2013) promises breeding success in terms of introgressing cold-tolerance genes into sugarcane (Głowacka et al., 2016).

Putative hybrids of *Saccharum* and *Miscanthus* have been studied since the late 1940s for their biomass production and adaptive traits (Li, 1948; 1961; Loh & Wu, 1949; Price, 1965; Chen & Lo, 1989; Xiao & Tai, 1994; Burner, 1997; Chen et al, 2000; Fageria et al., 2013; Głowacka et al., 2015). There have been reports confirming natural intergeneric crosses between *Saccharum* and *Miscanthus* (Price & Daniels, 1968; Grivet et al., 2006) as well as development of self-compatible intergeneric hybrids through artificial crossing between sugarcane and *M. sinensis* (Dong, 2017 ; Sharma, unpublished data). These hybrids, often termed as miscanes, show potential as lignocellulosic biomass crops with their strong, thick culms, long stems, and high biomass-yield potential (Sage et al., 2013; Sacks et al., 2013; Burner et al., 2017). Studies conducted in Arkansas, USA reported miscane genotypes yielding greater biomass compared to *M. ×giganteus*, *M. sinensis* Andersson, giant reed (*Arundo donax* L.) or the switchgrass (*Panicum virgatum* L.) cultivar 'Alamo' (Burner et al., 2009; 2015). Burner et al. (2009) also reported overwintering capacity of miscane in Booneville, Arkansas, USA, where winter minimum air temperature can go as low as -14 °C. Although, initially these intergeneric crosses were focused on introgression of genes for disease resistance into sugarcane germplasm (Chen et al., 1993), the preliminary results of these recent studies (Głowacka et al., 2016; Kar et al., 2019) have shown a possible introgression of cold tolerance traits from *Miscanthus* to sugarcane that could lead to greater biomass production under subtropical and warm-temperate latitudes (Głowacka et al., 2014; Sacks et al., 2013).

Given that *Miscanthus* and sugarcane each perform very differently in cool environments, the need exists to compare the seasonal variation in photosynthesis of miscane hybrids with their

respective parents to evaluate their capacity as a potential lignocellulosic biomass crop in higher latitudes. Although previous research indicated higher cold tolerance than sugarcane (Głowacka et al., 2016; Kar et al., 2019) and biomass yield capacity (Burner et al., 2009; 2015) in miscane genotypes, there has been limited work comparing their photosynthetic and biomass traits to their parental genotypes under subtropical and warm-temperate latitudes.

The present study evaluated miscane genotypes under warm-temperate summer conditions in northern Japan, and compared their photosynthetic rates with their respective parental genotypes and genotypes in related genera within the "*Saccharum* complex". In a 2-year field trial at Hokkaido University, Sapporo, Japan (43° 04′ N, 141° 20′ E), we sought to determine whether miscanes exhibit high photosynthetic rates early and late in the season and produce biomass relative to their sugarcane parents under warm-temperate conditions.

Materials and Methods

Plant material

Plant material for the experiment included two parental sugarcane genotypes ('KR 05-619', and 'KY 06-139'), two *Miscanthus* parents (*M. sacchariflorus* 'Miyakonojo', and *M. sinensis* 'Shiozuka'), 18 miscane F_1 progenies, two Korean *Miscanthus sinensis* hybrid accessions, and the *Miscanthus* ×*giganteus* 'Illinois' cultivar, and one wild sugarcane genotype (*Saccharum spontaneum*) (Table 4-1). The sugarcane parents were breeding lines developed at the Sugarcane Breeding Station, National Agriculture and Food Research Organization, Tanegashima, Japan (31° 44′ N, 131° 4′ E). The miscanes were bred by Yoshifumi Terajima at the Tropical Agricultural Research Front of the Japan International Research Center for Agricultural Sciences in Ishigaki, Okinawa, Japan. Two of the miscanes were derived from *M. sinensis*, and the remaining 16 were derived from *M. sacchariflorus*. All of the *Miscanthus* accessions used in this study were selections

from Hokkaido University. *Miscanthus sinensis* 'Shiozuka' was collected from Tokushima Prefecture, Japan (36° N, 138° E), and it was observed to be well adapted to Hokkaido (43° 04′ N, 141° 20′ E) conditions (data not shown). Both of the Korean *M. sinensis* hybrid accessions 'KMS079' and 'KMS080' were maintained at the experimental farm of Hokkaido University. *M. sacchariflorus* 'Miyakonojo' was collected from Miyazaki Prefecture, Japan (31° 43′ N, 131° 4′ E). In previous, unpublished work, all genotypes in this study have demonstrated sufficient over-wintering capacity in Hokkaido. The wild sugarcane (*Saccharum spontaneum*) accession was collected from a perennial stand maintained inside a high-tunnel at Hokkaido University Experimental Farm, where it had survived for 4 years without additional heating.

Experimental design and planting

The experiment was laid out in a randomized complete block design with twenty-six genotype, one plant per genotype per replicate, and three replications, blocked by years (2017 and 2018). For the experiment, all genotypes of miscane and sugarcane were propagated vegetatively from axillary buds on belowground stems at a field site at Hokkaido University, and then harvested and collected in 2016. Underground rhizomes of *Miscanthus* and *S. spontaneum* accessions were collected from pre-established stands in Hokkaido University Experimental Farm in October 2016. These underground stem and rhizome pieces were planted in plastic pots (dia. = 15 cm, h. = 15 cm, vol. = 2 L) in November 2016 in a temperature-controlled greenhouse at Hokkaido University. Before transplanting to the field in spring 2017, the aboveground stems of these potted materials were cut to 30 cm height and planted in the field on 19 May 2017. For field establishment in 2018, the underground plant materials were collected from the 2017 field experiment after harvesting the aboveground biomass in November 2017. The plant material was then maintained in pots over winter and transplanted in the field on 24 May 2018. In both years, the spacing within and between

rows was kept at 1 m and 1.5 m, respectively. The soil at the experimental site was Andisol with clay loam texture, which had a pH of 5.8 and an EC of 42.1 mS m^{-1} (Muchanga et al., 2017). The macronutrient contents of the soil were as follows: 3.1 mg NO_3^--N, 0.84 mg NH_4^+-N, 12.7 mg P_2O_5, 42.9 mg K_2O, 204 mg CaO, and 60.5 mg MgO per 100 g of top soil (0-15 cm) (Muchanga et al., 2017). Plants were watered every day by hand using a plastic hose for the first 2 weeks following transplanting. No fertilizer was applied to the field in both years. Weeds were killed by periodic mowing throughout the growing season.

Environmental monitoring

Data for mean monthly precipitation, maximum and minimum air temperature, and soil temperature were recorded continuously at 0.5 h interval throughout the growing season using a Model 900ET electronic weather station (Spectrum Technologies, Inc., Plainfield, Illinois, USA) located within 200 m of the study site (Fig. 4-1).

Photosynthetic, agronomic and biomass parameters

Photosynthetic gas exchange rate (A_n; µmol m^{-2} s^{-1}) was measured on the youngest, fully expanded leaves of each experimental unit, which was done at ambient temperature and Photosynthetic photon flux density (PPFD) on the youngest fully expanded leaves, determined by presence of ligule, on each plant in three replicates. During the measurement process, an individual leaf was enclosed in a controlled-environment cuvette of a steady-state photosynthesis system (LI6400XT, LI-COR Bioscience, Lincoln, NE, USA) at solar noon ± 2 h (10:00 am to 02:00 pm). Observations were taken three times during the treatment period (spring (second week of June), mid-summer (second week of August), and late fall (third week of October 2017, first week of November 2018). For each sampling observation period, 2 days were required for taking measurements.

Measurements on each experimental unit were randomized with respect to time over each 2-day measurement period.

Aboveground biomass of the plants was harvested on November 9 in both years. Plant stems were cut using a hand-held mechanical brush cutter (C282, Shindaiwa, Lake Zurich, IL, USA) at 15 cm height. Before cutting, the stalks of each plant were tied together with nylon strings to keep them from mixing with stalks of other plants while cutting. Only stems with hardened nodes were counted and recorded. A representative sample of 10 stems per plant per genotype were collected to estimate plant dry biomass. Stem length (cm), stem diameter (mm), number of nodes per stem, leaf length (cm), leaf width (mm) and number of leaves per stem were recorded from the representative sample. Based on the methods of Burner et al., (2009) each leaf was separated into leaf and stem (stem, leaf sheath, and senesced leaves) components. The separated leaf and stem components were dried at 60 °C in a drying oven and weighed to compute dry biomass for leaf and stem on a per plant basis.

Statistical analysis

Analyses of the data were performed with SAS procedure GLIMMIX (version 9.4, SAS Institute Inc., Cary, NC, USA). A linear mixed model with repeated measures and an unstructured covariance for the repeated measurements was fit to the data to compare seasonal variation in photosynthesis along with genotypic differences. A two-way ANOVA model was used to compare biomass parameters among genotypes. In both models the year of planting was included as a random effect. Means comparisons were implemented through the SAS LSMEANS statement with the ADJ=TUKEY option used to obtain a Tukey-Kramer multiple comparison adjustment of *P*-values for the differences of LS-means. In all cases, significance for comparisons was tested at $P \leq 0.05$.

A correlation analysis was implemented through the "pearson" method using "cor" function in R v 3.5.1 (R Core Team, 2015) to study correlation between photosynthetic and biomass traits. A scatter plot matrix showing the correlation coefficients between variables and the significance levels was made using the "PerformanceAnalytics" package in R v 3.5.1 (R Core Team, 2015).

Variance components of studied parameters were determined using "lme4" package of R v 3.5.1 (R Core Team, 2015). Analysis was done through a linear mixed model with all the terms of the model fitted as random effects:

$$Y = \mu + Year + Genotype + Genotype * Year + Error$$

Where, μ is the population mean.

Broad sense heritability (H_B^2) was estimated by the following formula:

$$H_B{}^2 = \frac{\sigma^2_{Genotype}}{\sigma^2_{Genotype} + \frac{\sigma^2_{Genotype*Year}}{2} + \frac{\sigma^2_{error}}{6}}$$

Where, $\sigma^2_{Genotype}$ is genotypic variance, $\sigma^2_{Genotype*Year}$ is Genotype × Year interaction variance and σ^2_{error} is error variance.

A principal component analysis (PCA) of a data set comprising seasonal photosynthesis, agronomic and biomass parameters was done using "PCA" function from "FactoMineR" package in R v 3.5.1 (R Core Team, 2015). Figures for the cluster and biplots were made using "factoextra" package in R v 3.5.1 (R Core Team, 2015). All other figures were made using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

Results

Seasonal photosynthesis

Analysis of 2 years of seasonal photosynthetic data showed a significant genotype × season interaction for observations taken in spring and late autumn (Fig. 4-2a, c). Although, photosynthetic rate was high in miscane, *Miscanthus*, and wild sugarcane genotypes compared to parental sugarcane genotypes in early spring, only miscane genotype 'JM 14-51' significantly differed from sugarcane genotype 'KY 06-139' (Fig. 4-2 a). Observations taken in the summer showed no significant differences between genotypes (Fig. 4-2b). However, wild sugarcane and miscane genotype 'JM 14-09' had relatively high photosynthetic rates (Fig. 4-2b). All of the genotypes showed significant reductions (59 to 83% across genotypes) in photosynthetic rate in late fall when compared to their respective summer values (data not shown). In late autumn, the highest photosynthetic rate was observed in *M* ×*giganteus* 'Illinois', which was significantly higher than both sugarcane genotypes 'KR 05-619' and 'KY 06-139' and miscane genotypes 'JM 14-06', 'JM 14-57' and 'JM 14-76' (Fig. 4-2c). All of the *Miscanthus* accessions photosynthesized at relatively high rates in late autumn, but miscane 'JM 14-09' had the highest CO_2 fixation rate among miscane genotypes. All miscanes, except for 'JM 14-06', 'JM 14-55', 'JM 14-57' and 'JM 14-76', photosynthesized at higher levels than their respective parental sugarcanes (Fig. 4-2c).

Agronomic characters and biomass partitioning

Significant genotypic variation was observed in all of the agronomic and biomass traits (Fig. 4-3a-g). Among the stem characters studied, sugarcane genotype 'KR 05-619' had the longest stem length (256 cm), which was significantly greater than miscane genotypes 'JM 14-59', 'JM 14-47', 'JM 14-66', 'JM 14-72', 'JM 14-76' 'JM 14-61', 'JM 14-63' and 'JM 14-06', and all of the

Miscanthus accessions (Fig. 4-3a). Among miscanes, 'JM 14-09' showed the longest stem length (250 cm), however it was not significantly different from other miscane genotypes (Fig. 4-3a). Similar to stem length, stem diameter was also observed to be greatest (~ 30 mm) in both sugarcane genotypes, followed by miscane 'JM 14-09' (22.7 mm) (Fig. 4-3b). The range of stem diameter observed was between 7.7 and 27.7 mm across all miscane genotypes (Fig. 4-3b). Miscane 'JM 14-66' had more stems (78.3 ± 5.5) per plant compared to both *Miscanthus* and sugarcane parental genotypes, whereas other miscanes did not differ significantly from either parent (Fig. 4-3c). Parental sugarcanes, along with *M.* ×*giganteus* 'Illinois' and miscane 'JM 14-50' had fewer stems (<24.8 ± 5.5) than other genotypes in the study. However, the difference was not statistically significant, except for 'JM 14-66' (Fig. 4-3c). Although *M.* ×*giganteus* 'Illinois' had one of the lowest stem counts per plant, its stems had more nodes relative to all other genotypes (Fig. 4-3d). Among leaf characters, miscane genotype 'JM 14-09' and its sugarcane parent 'KR 05-619' had the longest leaves (158 and 157 cm, respectively) (Fig. 4-3e). However, sugarcane genotype 'KY 06-139' had the widest leaves (43 mm) (Fig. 3f). *Miscanthus* ×*giganteus* 'Illinois' had the highest leaf count per stem (16.0 ±0.7) (Fig. 4-3f).

Significant genotypic variation existed for leaf, stem and overall biomass yield (Fig. 4-4). The ratio of dry leaf to stem dry matter was 1.5:1 for miscanes, while for *Miscanthus* accessions, it was 1:1 (data not shown). Miscane genotype 'JM 14-09' produced more leaf (~ 2,700 g plant^{-1}), stem (~ 2,300 g plant^{-1}) and whole plant dry biomass (~ 5,000 g plant^{-1}) than all other genotypes, except for sugarcane 'KY 06-139' (Fig. 4-4). Apart from 'JM 14-09', 'JM 14-88' and 'JM 14-64' had relatively high levels of biomass, which were significantly greater than their *Miscanthus* parent 'Miyakonojo' and were similar to their sugarcane parents ('KY 06-139' and 'KR 05-619', respectively) (Fig. 4-4). All the miscane genotypes, except for 'JM 14-09', 'JM 14-88', 'JM 14-

64', and 'JM 14-63' produced biomass that were not significantly different from either of their sugarcane or *Miscanthus* parents (Fig. 4-4). Biomass production of individual miscane genotypes was observed to be as much or even higher than their sugarcane parents (Fig. 4-4). The biomass of miscanes, when compared with that of their parental sugarcane and *Miscanthus*, our data confirmed that mean biomass of miscanes was in the intermediate range to both of its sugarcane and *Miscanthus* parents (Fig. 4-5) suggesting an absence of transgressive segregation for the biomass trait among the F_1 progeny.

Correlation between photosynthetic, agronomic and biomass traits

A correlation matrix obtained from observed parameters indicated that summer photosynthesis (0.477), stem length (0.774), stem diameter (0.663), leaf length (0.666) and leaf width (0.717) are traits that strongly influence overall biomass yield among the genotypes studied (Table 4-2, Fig. 4-S1).

Heritability of photosynthetic, agronomic and biomass traits

Quantitative variation for a trait could be due to genotypic differences, interactions between genotypes, and/or environment. High broad-sense heritability values observed for stem diameter (0.81 and 0.88, respectively, for half-sib and full-sib F_1), number of stems (0.67 and 0.56, respectively, for half-sib and full-sib F_1), leaf width (0.87 and 0.84, respectively, for half-sib and full-sib F_1), stem dry weight per plant (0.75 and 0.73 respectively for half-sib and full-sib F_1), leaf dry weight per plant (0.82 and 0.79 respectively for half-sib and full-sib F_1) and total dry matter per plant (0.78 and 0.74 respectively for half-sib and full-sib F_1), indicate that the observed variation is due more to genetics than the environment. Hence, phenotypic selection on the basis of these traits could be feasible. Although the remaining traits have shown medium to low

heritability values, it suggests that a considerable proportion of the total variance is heritable and effective selection can be done on the basis of these traits as well (Table 4-3).

Overall genotypic responses

Correlations between photosynthetic, agronomic and biomass traits and similarities between genotypes were analyzed using PCA (Fig. 4-6). The first component, which explained 39.3 % of the variation in the data set, was positively correlated with stem length, stem diameter, leaf length, leaf width, stem and leaf dry weight per plant, and overall dry matter yield. The second component, which explained 16.9 % of the variation, was positively correlated with number of nodes, number of leaves, spring and fall photosynthesis, and was negatively correlated with number of stems (Fig. 4-6). The genotypes clustered distinctly and differed mainly along the second component axis (Fig. 4-6). This indicates that the genotypes are mainly distinguished by early and late season photosynthesis, number of nodes per stem, number of leaves and number of stems per plant. The cluster of *M.* ×*giganteus* 'Illinois' stood out from the rest of the genotypes. Miscanes showed an intermediate cluster between sugarcane and *Miscanthus* parents.

Table 4-1 Genotypes used in the experiment in 2017-2018, which includes 18 genotypes of miscane, two sugarcane parents, two *Miscanthus* parents, two *Miscanthus sinensis* Hybrids, one giant miscanthus and one wild sugarcane genotype.

Genotypes	Description	Female parent	Male parent
JM 14-06	Miscane	KY 06-139	Msi 'Shiozuka'
JM 14-09	Miscane	KR 05-619	Msi 'Shiozuka'
JM 14-47	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-49	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-50	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-51	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-52	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-55	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-57	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-59	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-60	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-61	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-63	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-64	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-66	Miscane	KR 05-619	Msa 'Miyakonojo'
JM 14-72	Miscane	KY 06-139	Msa 'Miyakonojo'
JM 14-76	Miscane	KY 06-139	Msa 'Miyakonojo'
JM 14-88	Miscane	KY 06-139	Msa 'Miyakonojo'
KR 05-619	Parental sugarcane	-	-
KY 06-139	Parental sugarcane	-	-

Msa 'Miyakonojo'	Parental *Miscanthus sacchariflorus*	-	-
Msi ‘KMS079’	*Miscanthus sinensis* Hybrid	-	-
Msi ‘KMS080’	*Miscanthus sinensis* Hybrid	-	-
Msi 'Shiozuka'	Parental *Miscanthus sinensis*	-	-
M ×g 'Illinois'	*Miscanthus ×giganteus* ‘Illinois’	-	-
Wild SC	*Saccharum spontaneum*	-	-

Table 4-2 Pearson correlations between photosynthetic and agronomic traits, and dry-matter yield among genotypes (n = 26).

Traits	**Pearson's correlation coefficients**
Spring A_n	-0.225^{ns}
Summer A_n	0.477*
Autumn A_n	-0.015^{ns}
Stem Length	0.774**
Stem Diameter	0.663**
Number of Stems	0.284^{ns}
Nodes	-0.083^{ns}
Leaf Length	0.666**
Leaf Width	0.717**
Number of Leaves	0.021^{ns}

ns Not significant at a 5% level.

*Significant at 5% level.

**Significant at 1% level.

Table 4-3 Variance components (σ^2) estimated from restricted maximum likelihood (REML), and broad-sense heritability (H_B^2) estimates of CO_2 assimilation rates (A_n) at Spring, Summer and Autumn, stem length, stem diameter, number of stem, number of nodes per stem, leaf length, leaf width, number of leaves, stem dry weight per plant, leaf dry weight per plant and total biomass yield per plant for a miscane F_1 half-sib family (sugarcane 'KR 05-619' or 'KY 06-139 × *Miscanthus sacchariflorus* 'Miyakonojo') and F_1 full-sib family (sugarcane 'KR 05-619' × *Miscanthus sacchariflorus* 'Miyakonojo'). Plants were grown in a field over two years 2017-2018.

	Half-sib family (*n* = 16)				**Full-sib family (*n* = 13)**			
Trait	$\sigma^2_{Genotype}$	$\sigma^2_{Genotype \times Year}$	σ^2_{Error}	H_B^2	$\sigma^2_{Genotype}$	$\sigma^2_{Genotype \times Year}$	σ^2_{Error}	H_B^2
Spring A_n	9.10	10.21	18.89	0.52	9.13	10.75	16.62	0.53
Summer A_n	8.50	1.36	37.36	0.55	6.47	3.20	36.03	0.46
Autumn A_n	0.17	6.16	0.17	0.05	0.00	3.66	0.16	0.00
Stem Length	112.44	531.75	20.56	0.29	191.38	415.56	19.22	0.48
Stem Dia.	3.78	1.55	0.68	0.81	4.11	0.95	0.61	0.88
No. of stems	148.55	128.82	47.86	0.67	107.67	154.76	43.29	0.56
Nodes	0.58	0.98	0.13	0.53	0.68	1.05	0.11	0.56
Leaf Length	52.75	248.73	14.45	0.29	101.87	210.14	14.01	0.49
Leaf Width	37.93	10.30	1.67	0.87	30.50	11.56	1.40	0.84

No. of Leaves	0.04	1.59	0.02	0.05	0.44	1.10	0.01	0.44
Stem DW/plant	50417.00	20312.00	40079.00	0.75	45695.00	22134.00	35457.00	0.73
Leaf DW/plant	128084.00	43479.00	40850.00	0.82	114198.00	46831.00	41175.00	0.79
Total DW/plant	289627.00	116810.00	151750.00	0.78	243333.00	124596.00	142682.00	0.74

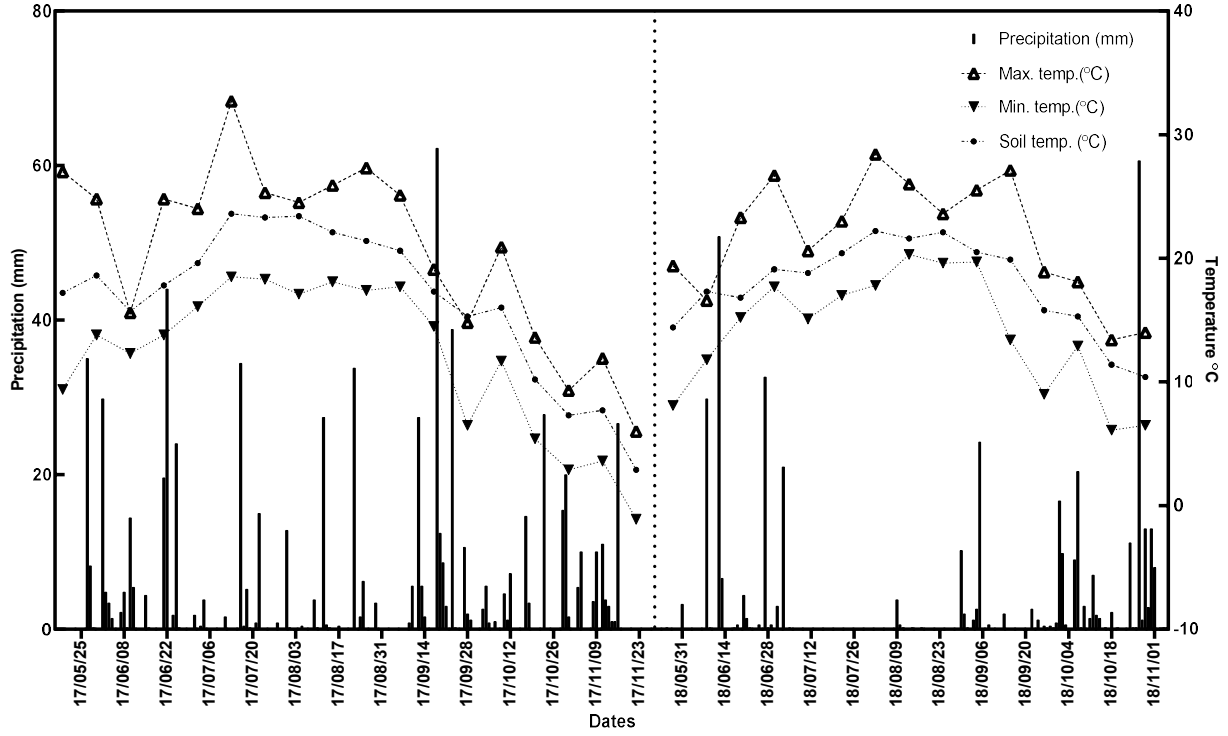

Fig. 4-1 Daily precipitation (mm), maximum and minimum temperatures (°C) and soil temperature (°C) observed *in situ* for cropping season in 2017-18. Experiment was conducted from 19th May till 9th November in 2017 and from 24th May till 9th November in 2018. Temperature data are shown at a 10 days interval

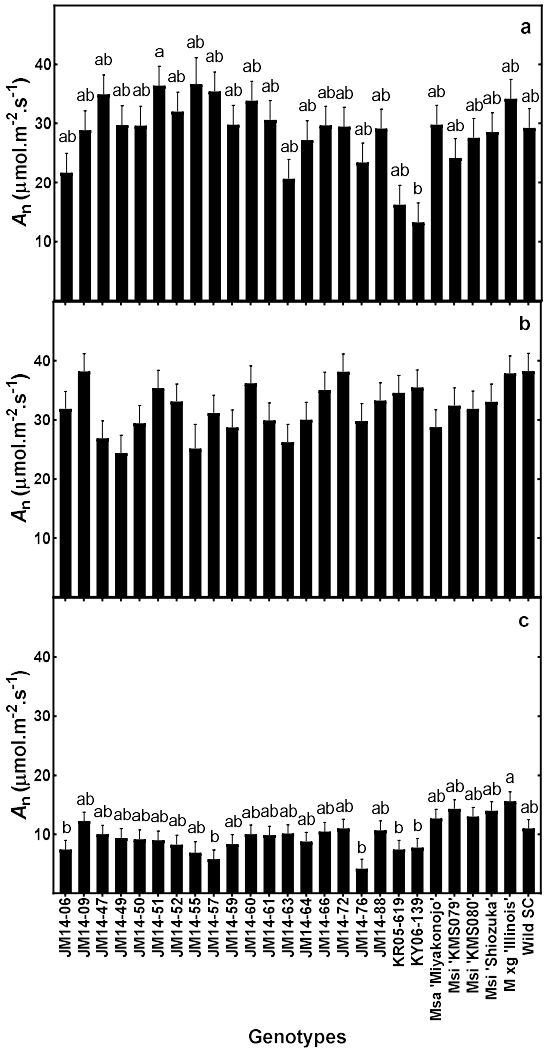

Fig. 4-2 Seasonal variation *in situ* gas exchange rate (A_n) at solar noon ± 2h. Bars represent least square means over two years of gas exchange rate observed in spring (a), summer (b) and autumn (c). Whiskers represent standard error of mean. Letters inside the panel on *X*-axis indicate comparison among genotypes within a particular season. A different letter indicates significant difference ($P < 0.0001$). Means separation was conducted by using the adjusted P-values from the Tukey-Kramer multiple comparison ($P \leq 0.05$). Spring: 2nd week of June; Summer: 2nd week of Aug.; Autumn: 3rd week of Oct. in 2017 and 1st week of Nov in 2018

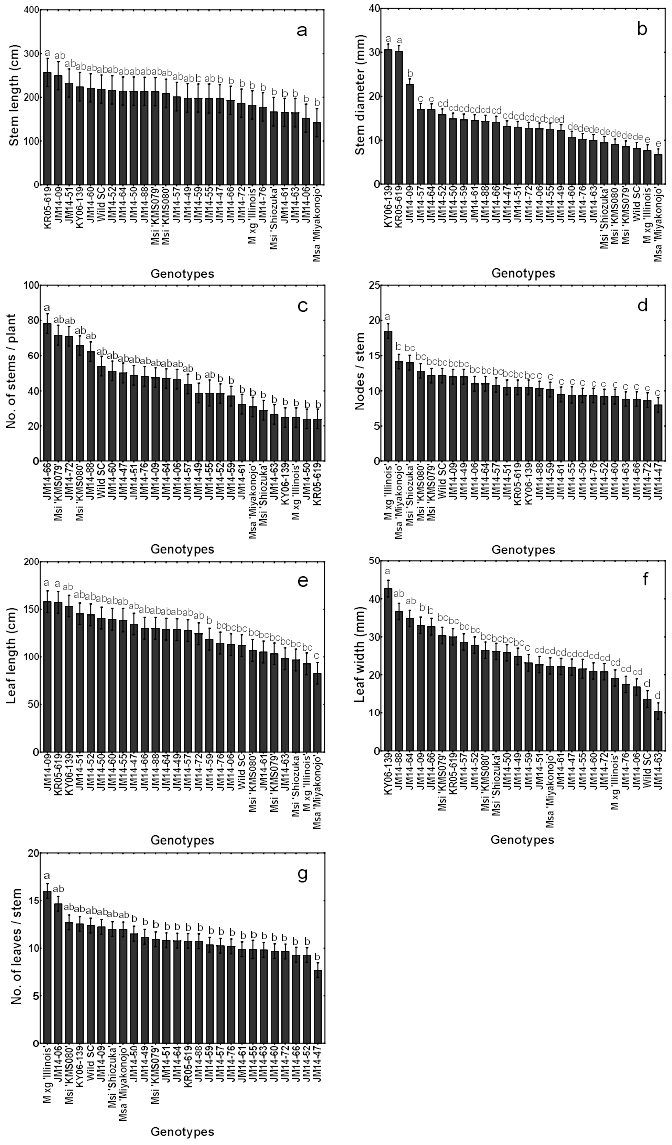

Fig. 4-3 Genotypic variation in stem length (a), stem diameter (b), no. of stems / plant (c), no. of nodes / stem (d), leaf length (e), leaf width (f) and no. of leaves / stem (g) after harvest. Bars represent least square means over two years. Whiskers represent standard error of mean. Letters indicate comparison among genotypes. A different letter indicates significant difference ($P < 0.0001$). Means separation was conducted by using the adjusted P-values from the Tukey-Kramer multiple comparison ($P \leq 0.05$)

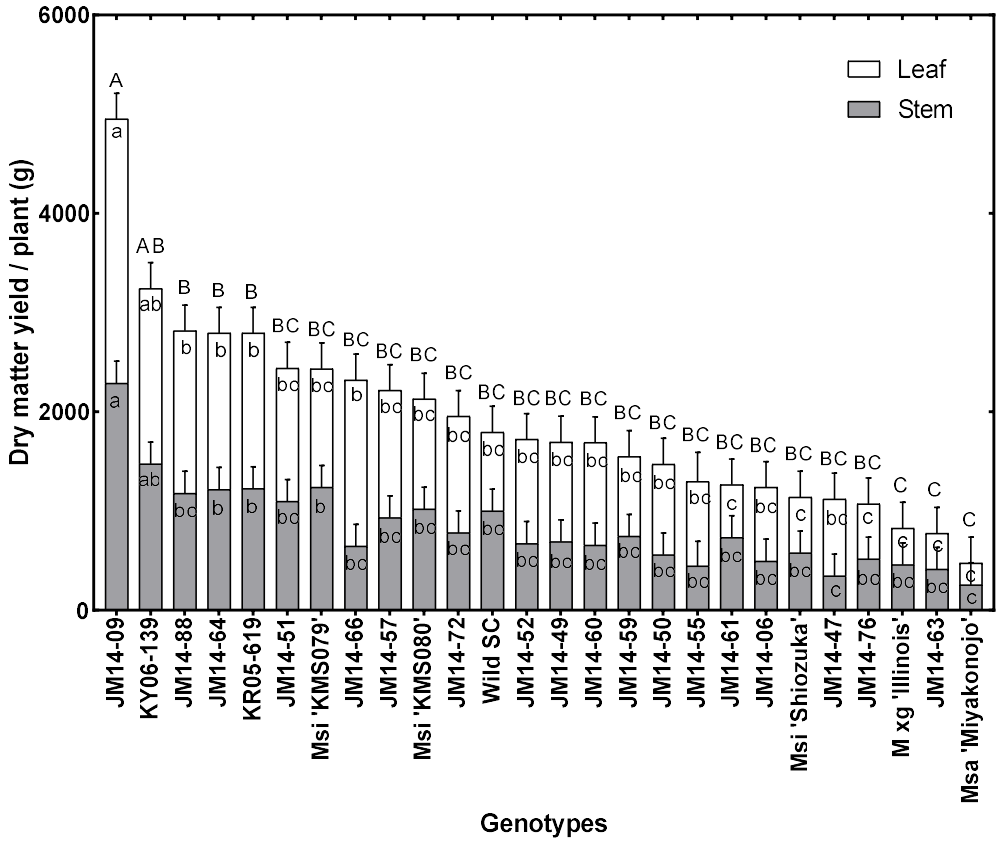

Fig. 4-4 Leaf, stem and above-ground biomass yield. Bars represent least square means over two years for each trait. Whiskers represent standard error of mean. Lower case letters indicate comparison among genotypes for leaf (white bars) and stem (gray bars) dry matter respectively. Upper case letter indicate comparison among genotypes for whole plant above ground dry matter yield. A different letter indicates significant difference ($P < 0.0001$). Means separation was conducted by using the adjusted P-values from the Tukey-Kramer multiple comparison ($P \leq 0.05$)

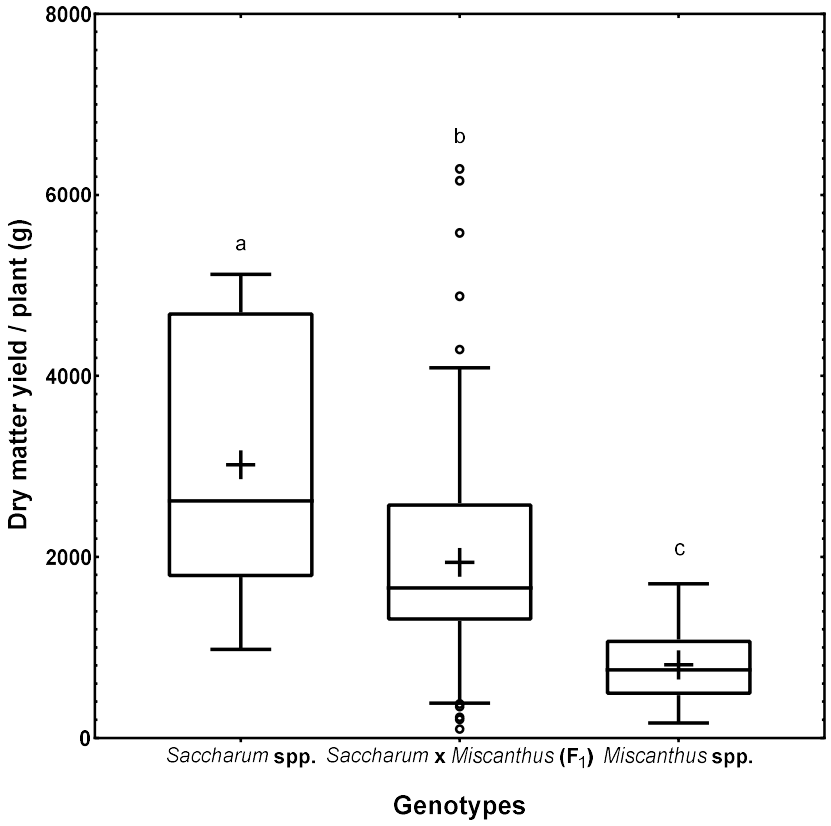

Fig. 4-5 Box plot, comparing plant cane biomass of parental sugarcane, *Miscanthus* and their hybrid miscanes. Horizontal lines inside the bars represent median and '+' signs represent means. Boxes represent the values between 1st and 3rd quartiles. Whiskers represent mid 90 percentile of values. Open circles represent outliers i.e., beyond 90 percentiles. Means were separated using Tukey's HSD test at $P \leq 0.05$. A different letter indicates significant difference at $P \leq 0.05$

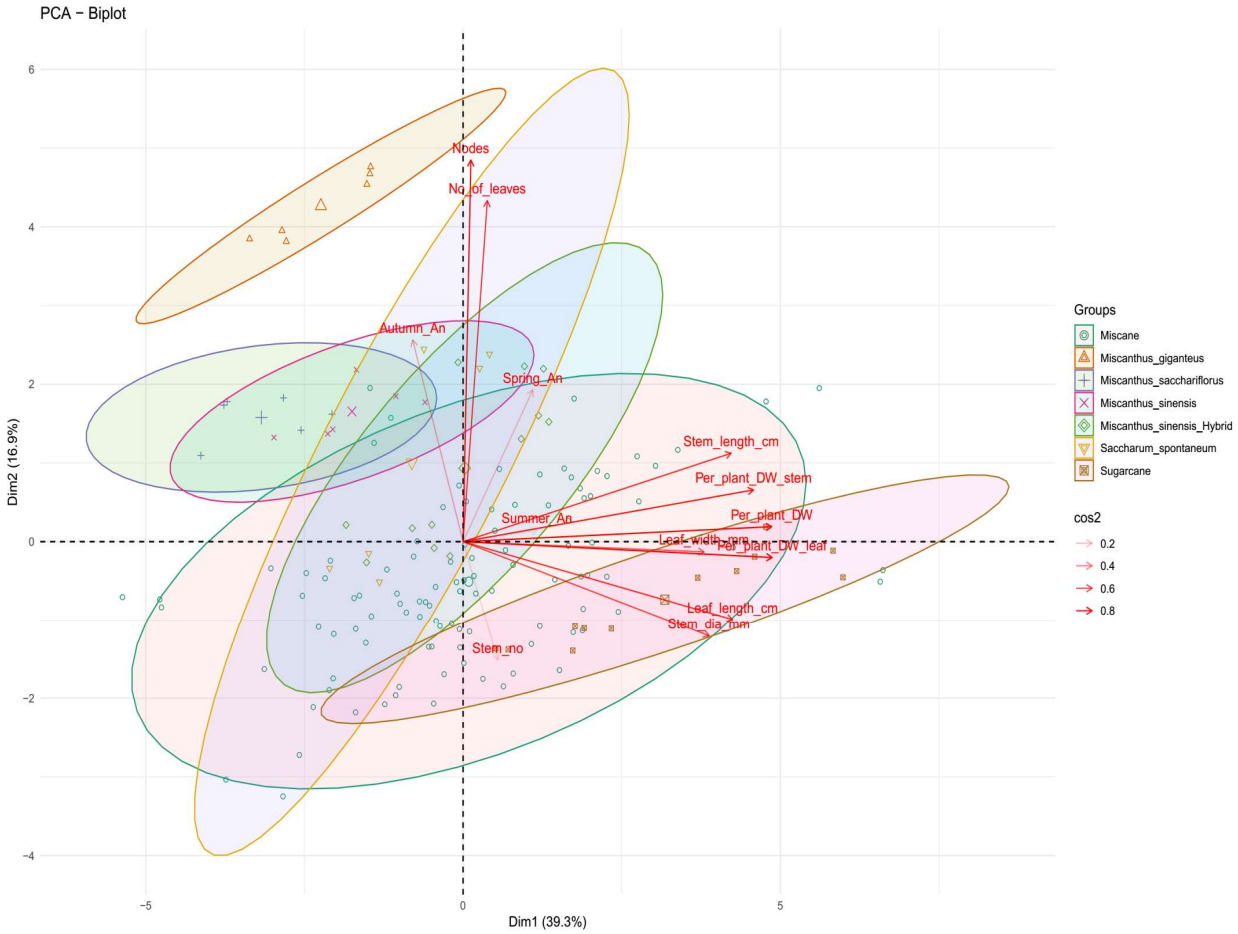

Fig. 4-6 Principal component analysis (PCA) grouping genotypes according to all measured parameters studied in 2 years of field experiment with biplot showing relationship between measured parameters. cos2 indicates a component's contribution to the squared distance of the observation to the origin or the relative importance of a principal component for a given observation

Discussion

Seasonal photosynthesis as an indicator of chilling tolerance

Temperature and light levels influence CO_2 assimilation rate under field conditions through their effects on stomatal regulation, light utilization and enzymatic processes. At high latitudes, the quantum efficiency of photosystem II (ϕ) acts as a principal determining factor for photosynthesis as the lack of alternative pathway for the utilization of light-generated reducing power in C_4 plants, make them more susceptible to chilling-induced photoinhibition especially at lower temperatures (Long, 1983). Chilling, especially in susceptible species, reduces the quantum efficiency of photosystem II through photoinhibition (Long, 1983; Baker et al., 1988). High efficiency of photosystem II thus indicates chilling tolerance and, in turn, higher levels of CO_2 assimilation early and late in the season compared to susceptible species. The perennial nature of a chilling-tolerant species, such as *Miscanthus*, ensures the rapid generation of the plant canopy in the spring and its maintenance late into the season, which allows it to maximize its capture of solar radiation through a prolonged growing season relative to chilling-sensitive species (Głowacka et al., 2013). Such a trait acts as a clear advantage for biomass production in higher latitudes. *Miscanthus ×giganteus* 'Illinois' has been studied for its ability to survive chilling temperatures (Long & Spence, 2013) and its ability to maintain high quantum efficiency of photosystem II and CO_2 assimilation rate at low temperatures. The seasonal variation in photosynthesis for *M. ×giganteus* 'Illinois' observed in our experiment coincides with the seasonal variation observed by Beale et al., (1996). Głowacka et al., (2016) reported photosynthetic rate of *M. ×giganteus* 'Illinois' and miscane in late October to be 13.1 ±0.4 μmol $m^{-2}s^{-1}$, which is consistent with our results. All of the miscane genotypes in our experiment, except for 'JM 14-06', 'JM 14-57', and 'JM 14-76', expressed a high rate of CO_2 assimilation early and late in the season, comparable to chilling-tolerant *M. ×giganteus* 'Illinois',

which is an indication of their ability to tolerate low temperatures. The genotypic variability observed among the miscane genotypes for early and late-season photosynthesis could be due to different levels of chilling tolerance contributed by their *Miscanthus* parents and/or interactions between genes from the *Miscanthus* and *Saccharum* parents (Głowacka et al., 2016). Desirable traits often get lost in progenies following interspecific hybridization (Głowacka et al., 2016) however, the high levels of photosynthesis during the summer ensures that their chilling tolerance does not come at a cost of reduced photosynthesis. Although the miscanes showed high early- and late-season photosynthesis, their failure to overwinter suggests that chilling-tolerant photosynthesis doesn't ensure overwintering (Głowacka et al., 2016).

Miscanes as potential biomass crops in higher latitudes

The overall morphology of miscanes used in our experiment was typical as previously described for by others (Artschwager, 1925). Significant genetic variability in stem length and stalk diameter in miscane genotypes have previously been reported (Burner et al., 2009). We also found genetic variability among miscane genotypes in all of the agronomic and biomass characters studied. Average stem length (2.1 m) observed for miscane accessions in our experiment was slightly lower than was previously reported for miscane (Burner et al., 2009; 2015). However, the best performing miscane in our experiment, 'JM 14-09', had a stem length (2.5 m) consistent with and a stem diameter (22.7 mm) greater than previously reported for miscane (Burner et al., 2009; 2015) and sugarcane (Viator & Richard, 2012). Miscanes out-yielding *M.* ×*giganteus* 'Illinois' at the plant cane stage had been reported previously (Burner et al., 2015) in terms of both leaf and stem dry biomass (Burner et al., 2009). Our results support those findings. However, per plant dry matter yield of the best performing miscane, 'JM 14-09' in our experiment was greater than previously

reported of 'US84-1028', a chilling-tolerant and high-biomass-producing miscane evaluated by Burner et al. (2009). Rapid establishment growth of miscane compared to the slower growth of *M.* ×*giganteus* 'Illinois' is due to their differential temporal dynamics of biomass accumulation (Burner et al., 2015). Burner et al., (2015) did not observe significant differences between leaf and stem dry weight in miscane in the establishment year. In contrast to their finding, however, miscanes in our experiment showed a dry leaf:stem ratio of 1.5:1 (data not shown). The higher leaf biomass value relative to that of stems observed in our experiment could be due to a shorter growing season in Hokkaido compared to west-central Arkansas, where the previous study on miscane by Burner et al., (2009) and (2015) was conducted, which limits sink capacity of photosynthates in producing stem tissue.

Miscane successfully overwintered in Booneville, Arkansas (35° 08′ N, 93° 98′ W), despite losing vigor and other agronomic advantages over *M.* ×*giganteus* 'Illinois' past the plant cane stage (Burner et al., 2015). Despite some of the miscanes showing high biomass yields at the plant cane stage, none of them survived winter conditions, suggesting that Hokkaido (43° 04′ N, 141° 20′ E) remains outside the cultivation zone for miscanes. Considering cultivation of sugarcane is largely limited below 30° N latitude, this is not a surprise. Maximum aboveground biomass of a typical perennial plant is achieved after several years of growth. However, high biomass production at the plant cane stage coupled with high early- and late-season photosynthesis in our miscane genotypes indicate a greater adaptability to cooler conditions in the early spring and late fall than sugarcane. As such, cultivation of miscanes at a lower latitude than Hokkaido should be feasible. Miscane genotype 'JM 14-09', which has phenotypic characters typical of elite sugarcane genotypes with large stalk diameter and wide leaves, offers potential to be selected as a cultivar in the coming years.

Correlation between biomass and photosynthetic and morphological traits

Głowacka et al. (2013) reported a high value of correlation (0.78) between summer photosynthesis and biomass yield in *Miscanthus*. The efficiency of *Miscanthus* to intercept and utilize solar radiation into biomass (0.06) is close to the C_4 maxima (Beale & Long, 1995; Dohleman et al., 2009) in cool environments, but for warm season grasses like sugarcane, the value would be much lower (Dohleman et al., 2009).Hence, the correlation value between the summer photosynthesis and biomass yield observed in our experiment is reliable. Several of the miscanes had high summer photosynthetic rate levels, but produced lower biomass, which indicates a variability in the capacity to convert solar-radiation into biomass. Hence, this trait could be exploited for selecting improved miscane genotypes. Significant positive correlation of stem length and stem diameter has previously been reported for giant grasses, such as *Miscanthus* and *Saccharum* (Pude & Jezowski, 2003; Jezowski, 2008; He et al., 2015). Leaf area growth determines the light interception capacity of a plant and is often used in high-throughput phenotyping systems as a replacement for plant growth (Weraduwage et al., 2015). The relationship between leaf area and dry matter depends on how carbon is partitioned among the above- and below-ground organs of the plant. A significant positive correlation between leaf length, leaf width and biomass observed in our experiment indicates a crucial relationship between leaf area and biomass. In addition, we observed 1.5 times as much leaf biomass in miscane in our experiment than stem biomass. Hence, the leaf organ itself is an essential biomass trait for miscane, apart from its role as photosynthetic machinery.

Selection of traits for improvement

Both the *Miscanthus* and sugarcane parents are highly heterozygous. Consequently, segregation among the F_1 progeny is expected. The success of a varietal improvement depends on the presence of genetic variability in the population. Although our study involved a small number of hybrid progenies and the heritability estimates should be interpreted with caution, our data is consistent with reports of high heritability values for stem number, stem diameter and single cane weight (Nair et al., 1980; Chaudhary, 2001; Jamoza et al., 2014; Tena et al., 2016). Knowledge of variability and heritability of characters is essential for identifying traits for selection (Vidya et al., 2002). Moderate broad-sense heritability of summer photosynthesis and its significant positive correlation with biomass can be a useful selection tool. Miscane genotype 'JM 14-09' demonstrated a high summer photosynthetic rate in addition to high biomass, which suggests that through careful selection, the capacity of miscanes to convert solar radiation into biomass can be improved. Stem length, stem diameter, leaf length, and leaf width had positive and significant correlation with final dry matter yield. The fact that these traits showed moderate to high broad-sense heritability suggests that they as well can be used successfully for future improvement programs. Khan et al. (2007) and Tena et al. (2016) reported that significant yield improvement is possible with selection based on these traits. Results of our study indicate that use of these high-heritability traits as selection criteria togethser with biomass yield could be a key aspect for genetic improvement in sugarcane.

Implication of breeding

As expected from our results obtained for the different parameters, miscanes took an intermediate position in the PCA plot. Analysis of the overall response of genotypes and observed traits indicated that early and late season photosynthesis was the main differentiating factor, with *M.*

×*giganteus* 'Illinois' clearly separated from rest of the genotypes, while all of the *Miscanthus* accessions showing distinct clustering from sugarcane. Wild sugarcane expressed a wide variability in the PCA plot, which indicates its uncertainty as a genetic resource for improving sugarcane for chilling tolerance (Burner et al., 2009). Chilling tolerance, like biomass is a complex combination of genetic and physiological traits, and it will likely be advantageous to do selection for different component traits when breeding sugarcane for adaptation to cooler environments than where this crop is currently grown.

Despite the potential of *Miscanthus* in broadening the genetic base and environmental adaptability of sugarcane, very little is known about the photosynthetic and agronomic characters of their intergeneric hybrid genotypes, and the relationships between them in cooler temperate environments. This lack of data therefore limits the utilization of *Miscanthus* as a genetic resource for improving sugarcane. Our experiment compared seasonal photosynthesis and biomass partitioning among *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes) with taxa belonging to the *Saccharum* complex, which included their sugarcane and *Miscanthus* parents. To the best of our knowledge, this is the first report to compare agronomic and photosynthetic traits of miscane hybrids with its respective parental species. Genotypic variability observed in photosynthetic, agronomic, and biomass traits among miscane genotypes, revealed key traits for selection to improve genotypes for better adaptability and biomass. We also identified a potential cold-tolerant and high-biomass-producing miscane, 'JM 14-09' which could be among the very first cultivated miscanes. Sapporo falls within USDA plant hardiness zone 7b (annual extreme minimum temperature between -15°C to -12.2°C), which is outside the conventional sugarcane cultivation range. Hence, overwintering was not observed in any of the miscane genotypes studied. However, our results strongly indicate that with careful selection, miscanes can become important

lignocellulosic biomass producers in sub-tropical and warm-temperate latitudes, where cultivation of sugarcane is limited by its physiological susceptibility to cold.

Chapter 5: General Discussion

Physiological susceptibility to chilling temperatures limits cultivation of sugarcane (*Saccharum* spp. hybrid), a major crop for lignocellulosic biomass, refined sugar, and bioethanol, to tropical and sub-tropical regions. Interspecific and intergeneric hybridization have been attempted to broaden the genetic base of sugarcane and improve its adaptation to temperate climates. Chilling tolerance can be introgressed into sugarcane through intergeneric hybridization with *Miscanthus*, a cold-tolerant C_4 perennial grass, which is genetically homologous to sugarcane. This creates an avenue in exploiting *Miscanthus* to broaden the genetic base of sugarcane and improve its adaptation in warm-temperate regions of the world. The hybrids of *Saccharum* and *Miscanthus* can be potential biomass producers in subtropical and warm-temperate regions. To achieve this goal, it is necessary to establish a reliable selection methodology based on the genotypic variability in chilling tolerance and biomass production traits of these *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes). In this chapter the author discusses the following points: (i) Chilling tolerance ability of *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes), based on the results given in chapter 2 to 4; (ii) Recovery from chilling damage; (iii) Miscane as a source of biomass in warm-temperate regions of the world; (iv) Genetic control of chilling tolerance and selection of improved genotypes.

Evidence of chilling tolerant photosynthesis in miscanes

The current study revealed evidence of chilling tolerant photosynthesis in *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes). It has been shown that the studied 18 miscane genotypes, derived from hybridizations between two genotypes of sugarcane and two genotypes of *Miscanthus* (one each of *M. sinensis* and *M. sacchariflorus*), in both greenhouse, growth

chamber and field conditions show a varying degree of chilling tolerance (Chapters 2 to 4). The chilling tolerance trait was dependent on the temperature and duration of chilling in miscane genotypes (Chapters 2 and 3). Experiment on long-duration chilling stress revealed that photosynthetic rates of several miscanes were higher than their sugarcane parents after 7, 14 and 23 days of chilling, and some of these did not differ from their highly tolerant *Miscanthus* parents (Chapter 2 and 3). Evaluation under short-duration chilling stress for 1, 3 and 4 days revealed that miscanes retained more of their pre-chilling photosynthetic rates compared to their sugarcane parent (Chapters 2 and 3). Even when the plants were grown under lower growth temperature, the photosynthetic rate in miscanes were higher than their sugarcane parents (Chapter 3). This short- and long-duration chilling stress tolerance in miscane was also realized under field conditions where several of the miscane genotypes showed high early- and late-season photosynthesis (Chapter 4). Hence the authors were able to confirm the presence of chilling tolerance in miscanes.

Typically, under subtropical and warm-temperate environments early and late season temperature variation is common. This, along with high intensity of sunlight can have lasting damage to photosynthetic apparatus of a C_4 plant (Long, 1983). Chilling tolerant photosynthesis thus is essential for successful establishment and production of a crop under these conditions. The results of the present study indicate that miscanes are capable of maintaining and retaining a high photosynthetic rate under different degrees and duration of chilling stresses. The pattern of change in photosynthetic rate under chilling observed in these experiments has been reported for *Miscanthus*, sugarcane and miscane previously (Głowacka et al., 2016). Thus it is evident that genes from *Miscanthus* have improved chilling tolerance of sugarcane via introgression. Often when crossing a temperate-adapted species such as *Miscanthus* with a tropical adapted species like sugarcane for introgression of chilling tolerance traits, there is a potential tradeoff associated

(Głowacka et al., 2016). However, in these experiments the miscanes performed as good as their sugarcane parents under warm temperatures (Chapters 2 to 4) hence, no tradeoff for photosynthesis in miscanes was observed. This is a first published study which identifies and compares chilling tolerance ability in *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes) under both control-environment and field conditions. Although not all of the miscanes tested in these experiments showed chilling tolerance, it is evident that through careful selection the chilling tolerance traits in miscanes can be improved.

Recovery from chilling stress

Recovery from chilling stress is one important aspect in terms of success of a crop under cooler environmental conditions. Degree and duration of chilling determines the rate of recovery (Pignon et al., 2019). In the current experiments the author has shown that after short- and long-term chilling stress, miscanes recover relatively better after rewarming treatment compared to their chilling sensitive sugarcane parent (Chapter 3). However, the difference in recovery of pre-chilling photosynthetic rate is dependent mainly on the duration of chilling. The author observed that short-term chilling (3 days) followed by rewarming had little effect in loss of photosynthetic rate in any of the tested genotypes. However, long-term chilling (7 days) had lasting impact on CO_2 assimilation rate especially on chilling sensitive sugarcane, which could not recover even after 7 days of rewarming treatment (Chapter 3). Miscanes showed quick recovery both after short- and long-term chilling treatment. Moreover, the CO_2 assimilation rate achieved by some of the miscane genotypes during recovery were higher than their pre-chilling rates, which is another important indicator of their chilling tolerance nature (Chapter 3). Long warm periods following a chilling event is unlikely during spring and autumn seasons under high latitudinal conditions, hence quick

recovery of pre-chilling photosynthetic rate is necessary to utilize the short warm periods following chilling to maximize biomass production (Pignon et al., 2019). Miscanes performing as good as their chilling tolerant *Miscanthus* parents under chilling stress and recovering rapidly during rewarming period are novel findings in this study, which further confirms the potential of miscanes as biomass producers in cooler regions of the world.

Miscane as a source of biomass in cooler environments

This study evaluated the biomass production capacity of miscane genotypes under warm-temperate latitudinal condition of northern Japan which are well beyond the conventional sugarcane production range (Chapter 4). Results have shown genotypic variability in morphological and biomass characters of miscanes, some of which performed as good as their sugarcane parent. High biomass yield on first year at plant-cane stage in some of the miscanes indicate their likeness to elite sugarcane genotypes, that are high biomass producers in warmer regions of the world. This character combined with chilling tolerance is a promising indicator of miscanes' ability in broadening the sugarcane biomass production range in higher latitudes. The author has identified strong correlation between biomass and morphological traits such as stem diameter, tiller number, leaf width, leaf and stem dry weight. Surprisingly these traits also showed medium to high broad sense heritability which indicates that efficient selection can be made based on these traits to improve sugarcane. The author also has identified one promising miscane genotype 'JM 14-09' as a potential cultivar due to its chilling tolerance nature (Chapters 2 and 3) and high biomass production capacity (Chapter 4). This novel finding will be important in future breeding programs for improving sugarcane biomass production in northern latitudes of the world.

Genetic control for chilling tolerance and biomass traits in miscanes

The primary source of germplasm to improve sugarcane for tolerance to abiotic stresses have been *S. spontaneum* (Głowacka et al., 2016). However, the potential of *S. spontaneum* for breeding chilling tolerance in sugarcane is limited compared to *Miscanthus* owing to the more northern distribution range of *Miscanthus* compared to *S. spontaneum*. Therefor the hybrids between *Saccharum* and *Miscanthus* are assumed to be more chilling tolerant than sugarcane. There have been scarce reports on using *Miscanthus* to improve chilling tolerance in sugarcane. However, in the current study the author has successfully shown that chilling-tolerant photosynthesis of *Miscanthus* can be transferred into sugarcane via introgression. Genotypic variability in response of the miscane F_1 progenies to chilling temperatures was observed in the current experiment (Chapters 2 to 3). However, no transgressive segregation in chilling tolerance photosynthesis (A_{1000}) or dark-adapted chlorophyll fluorescence (F_v/F_m) was observed in miscane genotypes (Chapter 2). For A_{1000} on day 14 of chilling, the histograms for the F_1 miscane full-sib and half-sib families show an approximately normal distribution, which suggests that the *Miscanthus* and sugarcane parents each contributed alleles with predominantly additive effects from multiple loci that conferred chilling tolerant photosynthesis to their progeny(Chapter 2). In contrast to A_{1000}, histograms for F_v/F_m on day 14 of chilling showed a distribution skewed towards the resistant parent, which suggests that most if not all alleles for chilling-stability of photosystem II came from the *Miscanthus* parent (Chapter 2). In addition to chilling tolerant photosynthesis, the author has shown that the dry biomass of miscane F_1 progenies are in the intermediate range to both of its parent sugarcane and *Miscanthus* (Chapter 4). Previous reports on *Saccharum* × *Miscanthus* have also shown that agronomic traits in miscane hybrids are in the intermediate range to sugarcane and *Miscanthus* (Chen, 1953; Chen et al., 1983). Thus it suggests that like photosynthesis, biomass yield also is a product of additive gene effect from *Miscanthus* and sugarcane.

Although the author has provided a complete study which have identified chilling tolerance ability in *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes) and their biomass production capacity in warm-temperate latitudinal conditions of northern Japan, the following points need to be addressed for a clearer understanding of the limitations of this study. The greenhouse experiment described in Chapter 2 was conducted during winter season in Sapporo. Hokkaido received a total of ~280 mm of precipitation in snow during Dec. 2016 - Jan. 2017 along with ~80 hours of average monthly sunshine. Short days with scarce sunlight owing to overcast conditions are common occurrence during this period. Hence, the light intensity available for conducting the experiment was below the light-intensity to saturate C_4 photosynthesis, however, the author has shown that the photosynthetic response observed during chilling treatment followed a normal pattern i.e., sugarcanes performed at a lower, *Miscanthus* at higher and miscanes intermediate in CO_2 assimilation rate. Hence, the experimental technique can be trusted. In addition, the main limitation of this study was the field experiment where the author has reported only the first year of growth replicated over two years. This limitation is due to the climatic conditions of Hokkaido (USDA plant hardiness zone 7b, annual extreme minimum temperature between -15°C to -12.2°C), which is outside the conventional sugarcane cultivation range, where none of the sugarcane and miscane genotypes survived over winter. However, this study was able to successfully demonstrate genotypic variability in morphological and biomass traits and identified crucial selection parameters for breeding improvement of sugarcane. In the next section some future aspects are discussed based on the current research.

Future aspect

1. Molecular mechanism of chilling tolerance in miscanes

The molecular mechanism by which chilling-tolerant C_4 species, such as *Miscanthus* maintain high CO_2 assimilation rates under low temperature remains unclear. Chilling-induced reduction in C_4 photosynthesis have been correlated with: Rubisco activity (Kingston-Smith et al., 1997; Du et al., 1999; Pittermann & Sage, 2000; Chinthapalli et al., 2003), decreases in carboxylation efficiency via phosphoenol pyruvate carboxylase (PEPc) (Kingston-Smith et al., 1997; Chinthapalli et al., 2003), capacity for PEP regeneration via pyruvate orthophosphate dikinase (PPDK) (Du et al., 1999a) or a combination of these. C_4 plants have small spatial capacity for Rubisco due to their Kranz anatomy therefore, are inherently limited by Rubisco at low temperatures (Wang et al., 2008a). At temperatures below 20 °C photosynthetic rates shows the same pattern of decline as maximum Rubisco activity in chilling-tolerant C_4 species (Kubien & Sage, 2004; Pittermann & Sage, 2000; Sage, 2002). Rubisco large and small subunits (RbcL and RbcS respectively) play an important role in activity of Rubisco, and has an influence on photosynthesis of a plant (Spreitzer, 1993; Spreitzer & Salvucci, 2002). Therefore, it can be assumed that changes in expression of Rubisco genes in response to environmental stimuli may explain the molecular adaptation or acclimation of C_4 plants to low temperature (Yamori et al., 2006). A preliminary experiment conducted by the author have revealed that expression of Rubisco genes has significant positive correlation with CO_2 assimilation rate in sugarcane, miscane and *Miscanthus* genotypes (data not shown). Under chilling treatment sugarcane shows low CO_2 assimilation rate correlated with a low expression of both RbcL and RbcS, whereas *Miscanthus* shows high CO_2 assimilation correlated with high RbcL and RbcS expression and miscanes are intermediate in both (data not shown). The larger variation in expression of RbcS compared to RbcL observed in this experiment has previously been reported (Stein et al., 1990; Spreitzer, 1993). This suggests that future research should focus on evaluating expression patterns of Rubisco genes with respect to chilling that can

reveal the molecular mechanism of chilling tolerance in miscanes. In addition to Rubisco, PPDK is another key limiting factor for C_4 photosynthesis at low temperature (Wang et al., 2008a). PPDK catalyzes the regeneration of PEP as the primary acceptor of atmospheric CO_2 from pyruvate, ATP, and phosphate in C_4 photosynthesis (Du et al., 1999). PEPc is also known to play a key role in the initial CO_2 fixation in C_4 photosynthesis (Wang et al., 2008a). The PEPc gene influences the density and area of stoma in the leaves and in accumulation of dry matter in plants (Lian et al., 2014). Therefore, future researchers studying PPDK and PEPc genes can reveal key mechanisms of chilling tolerance photosynthesis in miscanes.

2. Miscane as a perennial biomass producer

As discussed earlier the main limitation of this study was that the miscanes and sugarcane genotypes did not survive over winter in the experimental site in Sapporo. Hence, the author could not determine the perennial biomass production capacity of miscanes. Considering the high latitudinal conditions of Hokkaido (43° N), this is not a surprise. Howsever, the success of a perennial biomass producer depends on its ability to overwinter and maintain high biomass production for several years without the need of replanting. Perennial biomass producers like *Miscanthus* achieve their peak biomass after 3-4 years of growth. Burner et al. (2015) reported miscanes surviving at a latitude 35° N in Arkansas and the present study also strongly suggests that miscanes used in the experiment can survive and produce high biomass at a lower latitude to Sapporo. Hence, future research should focus on determining the perennial biomass production capacity of miscanes as a potential biomass energy crop in cooler regions of the world.

3. Energy value of miscane as a source of biomass

For a lignocellulosic energy crop to be a successful feedstock, the energy output of its biomass is necessary to be high. Burner et al. (2017) had reported high *in vitro* digestible dry matter (IVDMD), total non-structural carbohydrate, hemicellulose and low combustible energy in leaf and stem tissues of miscanes. However, further research is necessary to evaluate the energy value of biomass components as well as the quality of the feedstock as a potential source of livestock feed.

4. Introgression breeding approaches

As mentioned earlier, introgression breeding through backcrossing often lead to a tradeoff for a potentially important trait (Głowacka et al., 2016). As the chilling tolerance trait introgressed into sugarcane from *Miscanthus* is dependent on the number of genes that confer the trait as well as their interaction with sugarcane genes (Głowacka et al., 2016), whether repeated backcrossing with sugarcane can be used without losing the favorable chilling tolerance trait is also a matter of crucial future aspect of research.

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Appendix I: Supplementary information of chapter 2

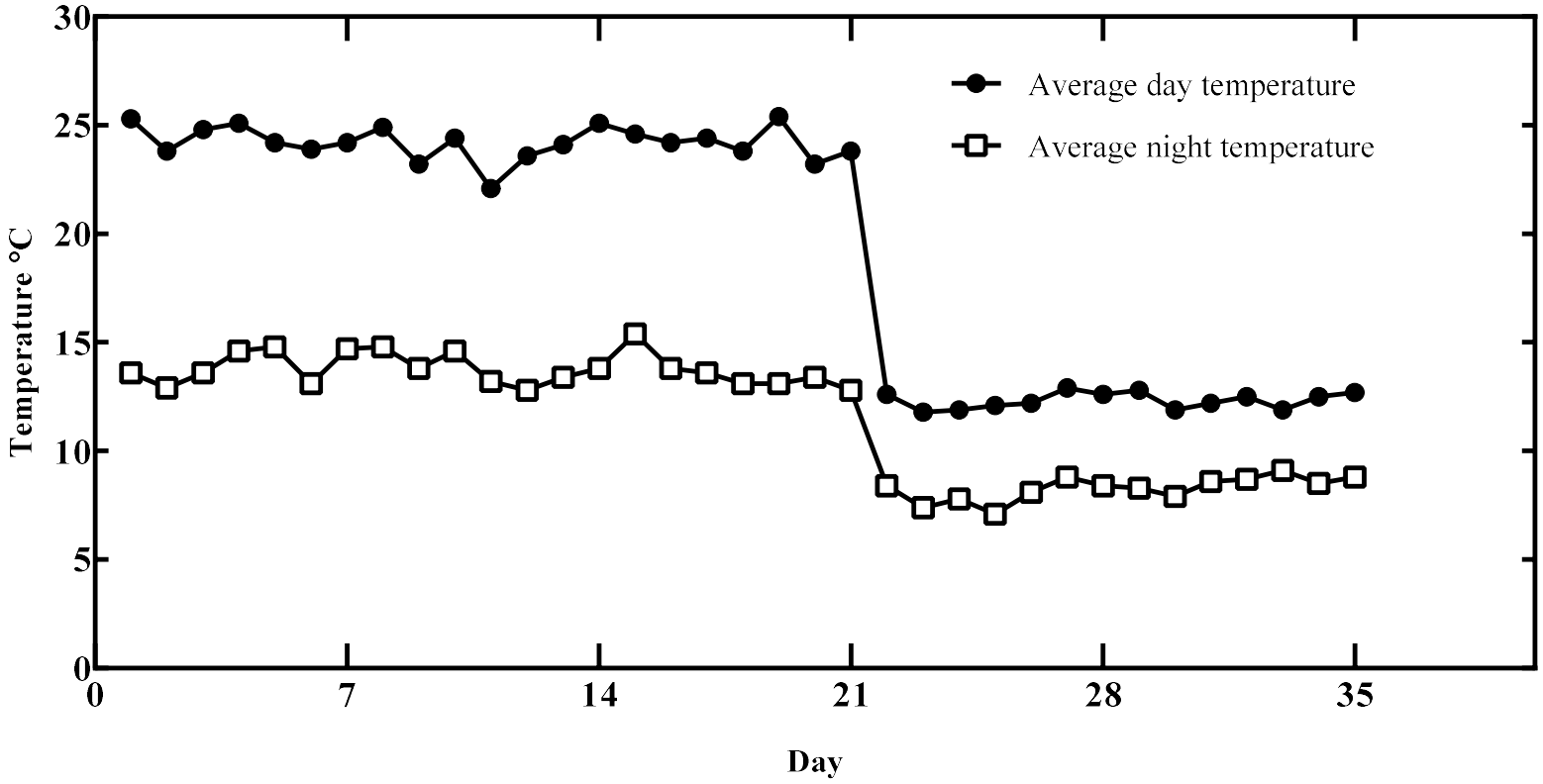

Fig. 2-S1 Daily average day and night temperatures (°C) in the greenhouse throughout the experimental period. First 3 weeks (21 days) are control, followed by cold treatment for 2 weeks (14 days).

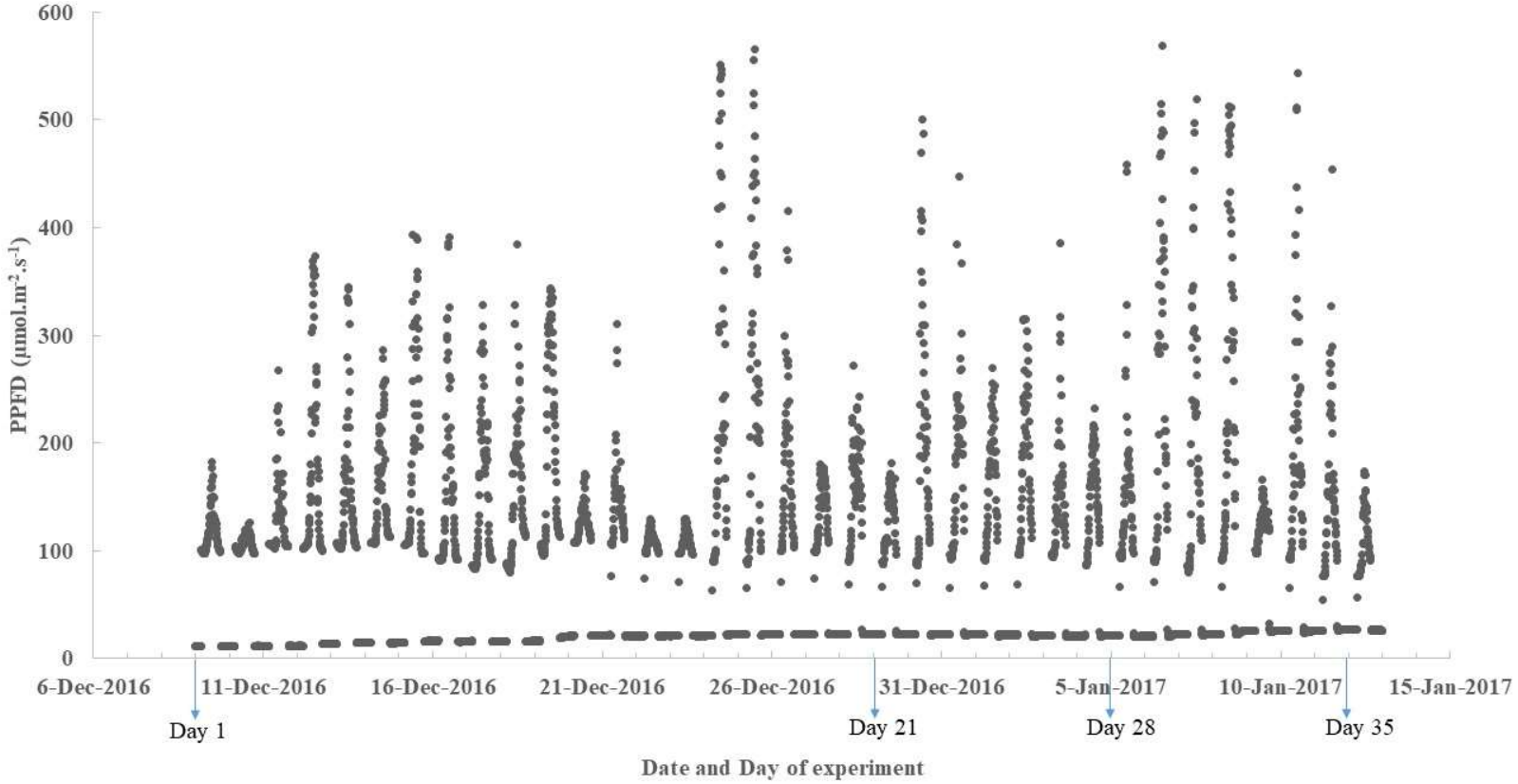

Fig. 2-S2 Photosynthetic photon flux density (PPFD) (µmol m^{-2} s^{-1}) in the greenhouse throughout the experimental period. Data are shown in 10 min. interval. First 3 weeks (21 days) are warm growth, followed by cold treatment for 2 weeks (14 days).

Table 2-S1 Variance components estimated from restricted maximum likelihood (REML), and broad-sense heritability (H^2) estimates of net CO_2 assimilation rate at 1000 μmols m^{-2} s^{-1} photosynthetic photon flux density (A_{1000}) and maximum quantum yield of photosystem II of dark adapted leaves (F_v/F_m,) for a miscane F_1 half-sib family (sugarcane 'KR 05-619' or 'KY 06-139 × *Miscanthus sacchariflorus* 'Miyakonojo'). Plants were grown in a greenhouse initially at 22-25 °C/13-15 °C day/night for 21 days (warm control period), then challenged with 14 days of 12-13 °C/7-9 °C day/night (chilling treatment).

	Half-sib family (n = 16)					
	A_{1000}			F_v/F_m		
Source	Warm period	7th day of chilling treatment	14th day of chilling treatment	Warm period	7th day of chilling treatment	14th day of chilling treatment
Block	0.00	0.00	0.01	0.000003	0.000000	0.000000
Genotype	14.16	5.53	3.41	0.000059	0.000257	0.000313
Error	0.64	0.63	0.24	0.000016	0.000014	0.000022
H^2	0.96	0.90	0.93	0.79	0.95	0.93

Appendix II: Supplementary information of chapter 4

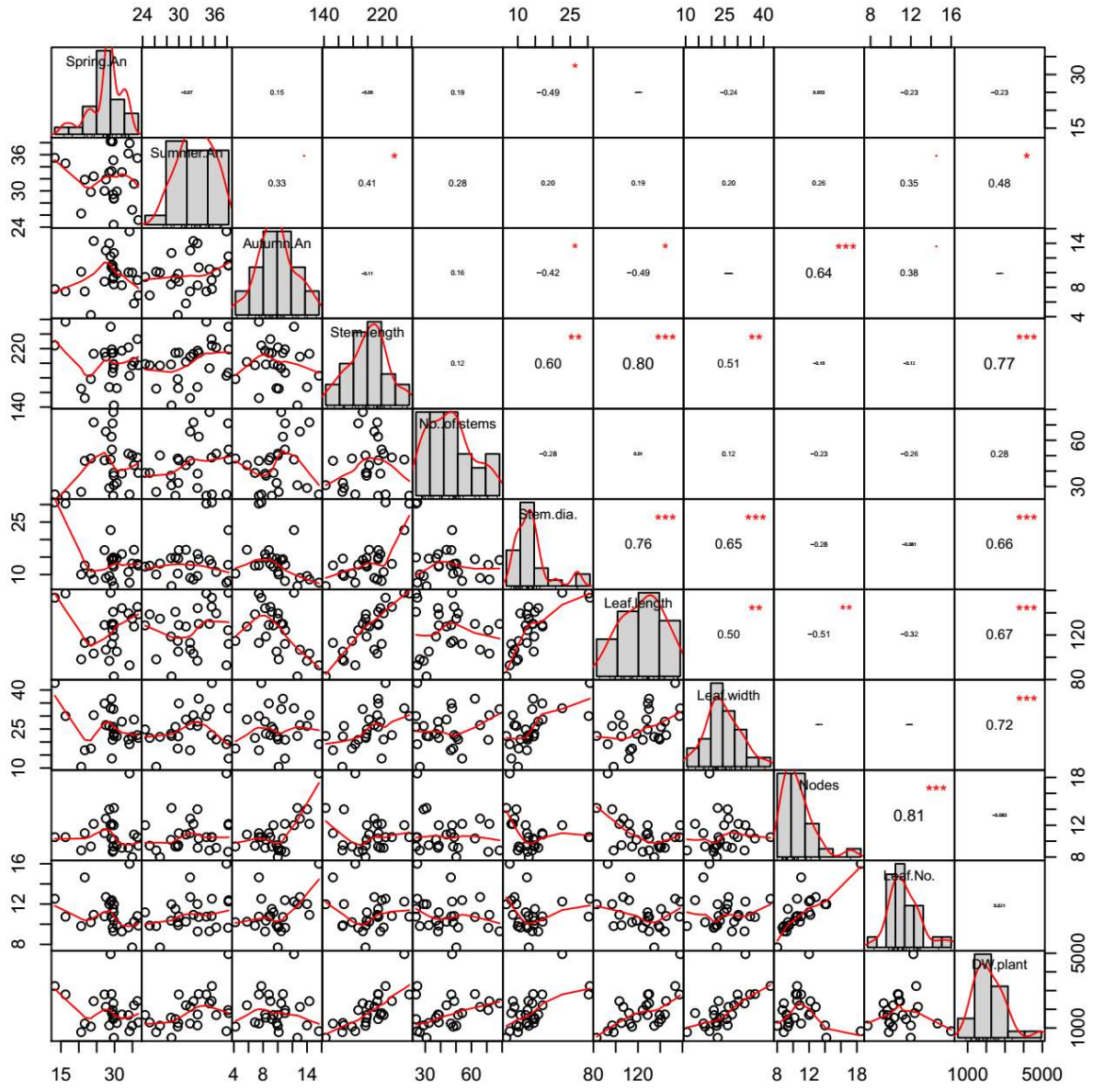

Fig. 4-S1 Pearson's correlation matrix among measured traits. Numbers on the right side of panels represent correlation coefficients between each pair of measured parameters. Significance is shown at P-values 0, 0.001, 0.01, 0.05, 0.1, 1 with symbols "***", "**", "*", ".", " " respectively

Summary

Biomass is the most common form of renewable resource that is abundantly used in the developing nations but not so much in the industrially developed nations. In 1992 at the Rio United Nations Conference on environment and development, the renewable intensive global energy scenario (RIGES) suggested that, by 2050, approximately 50% of the world's current primary energy consumption, could be met by biomass and 60% of the world electricity market would be supplied by renewables sources of which biomass is a significant part. Biomass is produced by green plants that convert sunlight into plant material through photosynthesis. 'Dedicated biomass energy crop' refers to nonfood crops that are solely grown for biomass production. Sugarcane (complex hybrids of *Saccharum officinarum* L., *Saccharum robustum* Brandes & Jeswiet ex Grassl, *Saccharum spontaneum* L., *Saccharum barberi* Jeswiet and *Saccharum sinense* Roxb. amend. Jeswiet), which is cultivated on 26.7 mha and yields nearly 1.9 billion metric tons per year with a peak dry matter yield >100 tons (ha yr^{-1}), is among the highest biomass-producing crops in the world. In addition to being the leading sugar-producing crop for human consumption, sugarcane is also used as a lignocellulosic biomass and a feedstock for bioethanol production. However, the lack of environmental adaptation of sugarcane has been a persistent problem, especially in high-latitude and/or high- elevation areas, owing to its susceptibility to cold. Sugarcane is one of the most chilling-sensitive crop species in the world. At temperatures below 20 °C, sugarcane leaf production slows, and below 10 to 15 °C growth ceases completely. Photosynthesis in sugarcane ceases between 8 to 12 °C and severe frost (-5 to -7 °C) can completely kill the aboveground plant. Hybridization programs have been attempted to improve cold tolerance and improved adaptation of this crop to cooler environments. Crosses between commercial sugarcane (hybrids of mainly *S. officinarum* and *S. spontaneum*) and *S. spontaneum* have been reported to show cold tolerance.

However, these crosses are not reliable for cultivation due to their varying degree of chilling tolerance. In addition to *S. spontaneum*, taxa in the *Saccharum* genus can be crossed with related genera belonging to so-called "*Saccharum* complex", which includes *Miscanthus*, *Erianthus*, *Narenga, Sclerostachya*, or other genetically similar perennial grasses. Among these related genera to *Saccharum*, *Miscanthus* is receiving a lot of focus in recent years as a potential genetic resource, especially to improve cold-tolerance in sugarcane. *Miscanthus*, a native C_4 grass of southeast Asia, unlike sugarcane, shows a high degree of cold tolerance compared to other warm-season, C_4 perennials. *Miscanthus* was reported to produce shoots at a temperature as low as 6 °C, and survive after prolonged exposure to temperatures <-6.5 °C. Putative hybrids of *Saccharum* and *Miscanthus* have been studied since the late 1940s for their biomass production and adaptive traits. These hybrids, often termed as miscanes, show potential as lignocellulosic biomass crops with their strong, thick culms, long stems, and high biomass-yield potential. Preliminary studies on miscanes have shown a possible introgression of cold tolerance from *Miscanthus* to sugarcane that could lead to greater biomass production under subtropical and warm-temperate latitudes.

Given that *Miscanthus* and sugarcane each perform very differently in cool environments, the need exists, 1) To observe genotypic variability in photosynthesis and biomass traits that will ensure important selection criteria to improve sugarcane; 2) To test chilling tolerance in miscane compared to its parent, sugarcane and *Miscanthus* which will confirm the introgression of chilling tolerance traits into sugarcane from *Miscanthus*; 3) To test the biomass production capacity of miscanes as a potential biomass energy crop in higher latitudes of the world.

Screening *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes) under low temperature

We studied 18 miscane genotypes, derived from hybridizations between two genotypes of sugarcane and two genotypes of *Miscanthus* (one each of *M. sinensis* and *M. sacchariflorus*). In a greenhouse experiment on long-duration chilling stress (12-13 °C day/7-9 °C night) photosynthetic rates of the *Miscanthus* parents were significantly higher than the sugarcane parents after seven days of chilling and were more than double by 14 days. The *Miscanthus* also retained more of their pre-chilling (22-25 °C day/13-15 °C night) photosynthetic rates (68-72% seven days, 64-66% 14 days) than the sugarcanes (27% seven days, 19-20% 14 days). Seven of 18 miscanes exhibited higher photosynthetic rates than their sugarcane parents after seven days of chilling, whereas after 14 days only four miscane genotypes had significantly higher photosynthetic rates than their sugarcane parents, but notably two of these did not differ from their highly tolerant *Miscanthus* parents. The results indicate variability in chilling tolerance in miscane progenies, thus selection will be a key aspect of improving chilling tolerance in sugarcane.

Chilling tolerance of *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes)

In three subsequent growth chamber experiments to evaluate chilling stress and post-chilling recovery, 4 miscanes representing the range of responses observed in the greenhouse experiment were compared with their parents. After short-term and long-term chilling stress of different temperatures, the miscanes retained more of their pre-chilling photosynthetic rate compared to their sugarcane parents, with some of the genotypes not significantly different from their *Miscanthus* parent and cold-tolerant *Miscanthus* ×*giganteus*. After one and seven days of post-chilling recovery, the *Miscanthus* genotypes and miscanes fully recovered their pre-chilling photosynthetic rates but the sugarcane parents did not. Confirming that genes from *Miscanthus* can be used to improve chilling tolerance of sugarcane via introgression.

Field performance of *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes)

This study evaluated intergeneric F_1 hybrids of *Saccharum* × *Miscanthus*, miscanes, for their seasonal variation in photosynthesis and biomass production under field conditions in Hokkaido, Japan to identify promising genotypes and traits, which can be selected to further improve sugarcane. Results show several of the miscane genotypes have high early- and late-season photosynthesis coupled with high biomass production, which likely indicates chilling tolerance. High broad-sense heritabilities for traits, including stem diameter, tiller number, leaf width, leaf and stem dry weight, and high correlations between these traits and dry matter yield indicate selections can be made efficiently to improve sugarcane. We identified miscane 'JM 14-09' as a superior genotype for introgression breeding programs and as a potential energycane cultivar for its high biomass-production capacity.

This is a first of its kind study which elucidates genotypic variability, chilling tolerance capacity and biomass production of *Saccharum* × *Miscanthus* intergeneric hybrids (miscanes) under warm-temperate climate of northern Japan, and compares it to its respective parental genotypes and taxa belonging to '*Saccharum* complex'. We identified chilling tolerant and high biomass producing miscanes that can be the very first cultivated miscanes. We also identified key traits for selection to improve chilling tolerance and biomass in miscanes. This study is an important database for future researchers interested in studying the use of sugarcane biomass in northern latitudes.

The main limitation of our study was the high latitudinal condition of Hokkaido (USDA hardiness zone 7b), hence, the overwintering capacity of miscanes could not be tested. The future research should focus on: 1) Perennial biomass production capacity of miscanes at lower latitudes

compared to Hokkaido; 2) The feedstock quality aspects such as cellulose, hemicellulose and lignin content for energy value in miscanes; 3) The genetic aspect of chilling-tolerance in miscanes, mainly focused on photosynthetic genes such as PPDK, Rubisco and PEPc, to get a clearer picture of the molecular mechanism of chilling tolerance observed in miscanes.

Acknowledgement

Firstly, I would like to thank my Ph.D. advisor Prof. Toshihiko Yamada for his supervision and guidance throughout my doctoral program at the Hokkaido University, Japan. I was deeply impressed with Prof. Yamada's meticulous attention to detail and pursuit of excellence, and such qualities, I believe, will be an asset to me in my future career. I thank my doctoral committee members: Prof. Hajime Araki, Prof. Toshihiko Hara, Dr. Yoichiro Hoshino and Dr. Taiken Nakashima for their insightful advice and helpful feedback. I acknowledge the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) scholarship to pursue my doctoral study at the Hokkaido University, Japan. I would also like to thank all the members in my lab for their expertise and invaluable help along every step of my research. I would also like to thank our collaborators: Prof. Ryan J. Stewart of Brigham Young University, USA, Dr. Erik J. Sacks of University of Illinois at Urbana-Champaign, USA, Dr. Na Zhang of China Agricultural University, China, Dr. Antonio Villanueva-Morales of Universidad Autónoma Chapingo, Mexico and Mr. Yoshifumi Terajima of Japan International Research Center for Agricultural Sciences, Japan. Thanks are in order to Mr. Rafael Alexandre Muchanga, Ms. Yufita Dwi Chinta, Ms. Zhihui Guo, Ms. Tzu-Ya Weng and Ms. Kiko Eguchi for help and advice on laboratory techniques, data analysis, field data collection, and trials maintenance. I also express my deep gratitude towards all the staff and technicians of our lab for their invaluable help during field and laboratory work. Lastly, I thank my parents for their support and love throughout my life. I thank my friends for their company and friendship throughout the years.