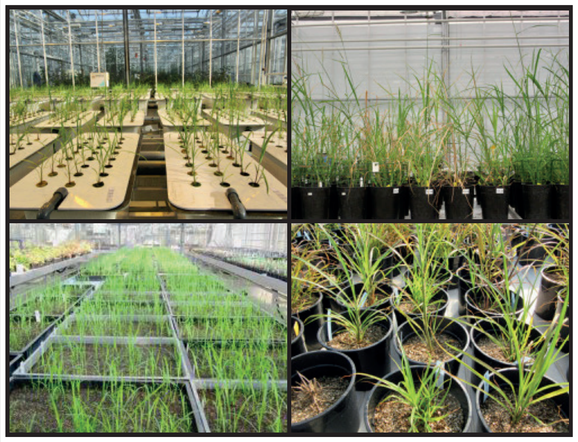

Genetic diversity and mechanisms of salt tolerance of *Miscanthus*

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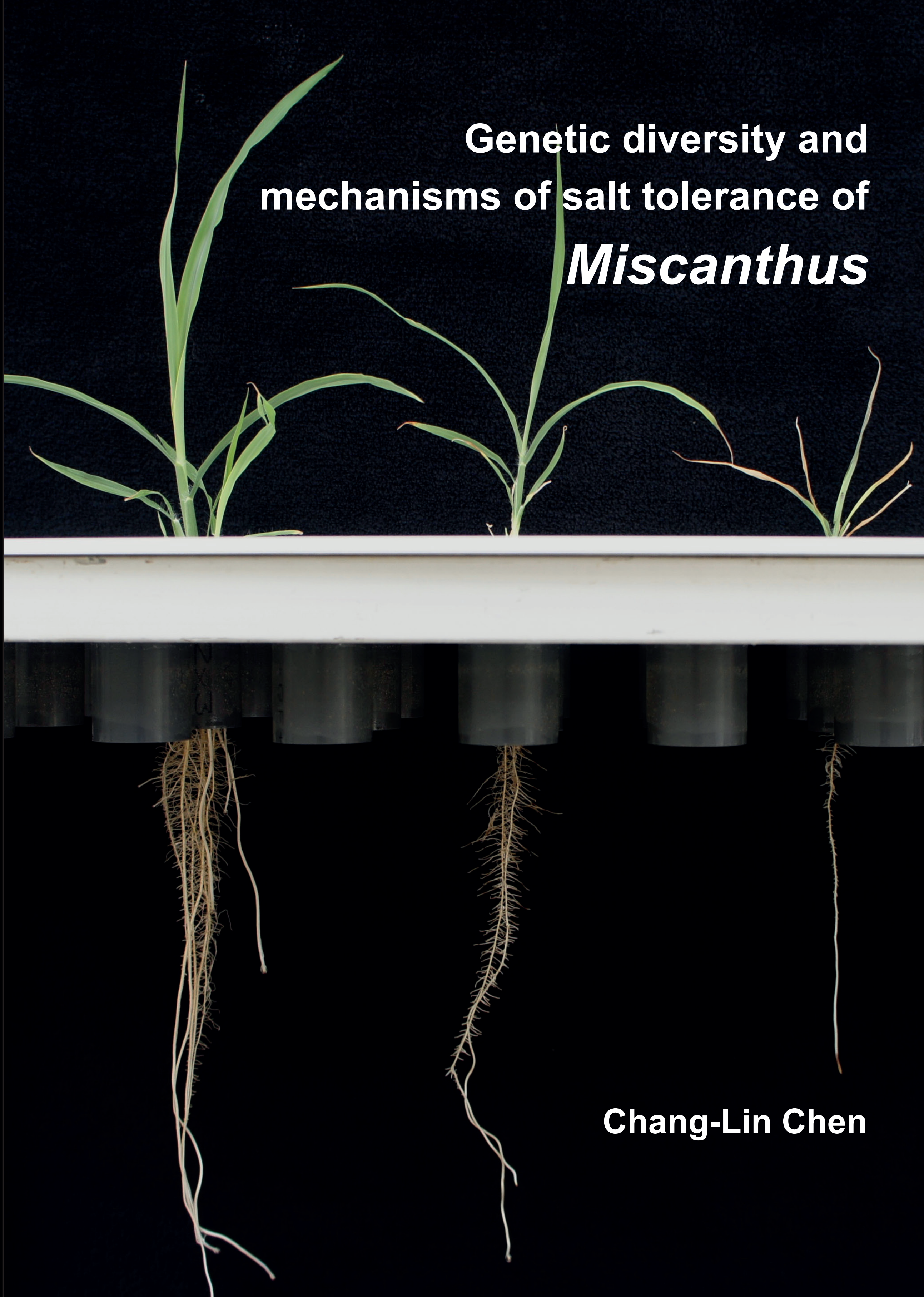

Genetic diversity and mechanisms of salt tolerance of *Miscanthus*

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Propositions

1. It is impossible to improve salt tolerance of *Miscanthus* without exploiting the genetic diversity of this plant species.
   (this thesis)

2. *Miscanthus* is an ideal crop to produce large amounts of biomass on marginal lands.
   (this thesis)

3. Bioenergy crops are more environmentally friendly than solar panels.

4. The biggest challenge for bioenergy crops is to produce additional value products.

5. Protection of ecosystems in marginal lands is better than utilizing them.

6. Gardening improves human health and social skills.

Propositions belonging to the thesis

"**Genetic diversity and mechanisms of salt tolerance of *Miscanthus***"

Chang-Lin Chen

Wageningen, 19 September 2018

Genetic diversity and mechanisms of salt tolerance of *Miscanthus*

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Genetic diversity and mechanisms of salt tolerance of *Miscanthus*

Chang-Lin Chen

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 19 September 2018
at 11 a.m. in the Aula.

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Genetic diversity and mechanisms of salt tolerance of *Miscanthus,*

194 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2018)

With references, with summary in English

ISBN: 978-94-6343-322-8

DOI: https://doi.org/10.18174/456351

Contents

Chapter 1

General introduction

1 Bioenergy crops

Using alternative energy sources to reduce carbon dioxide emission is an important policy in order to reduce global warming. With strong political support, the first-generation crops successfully produced different types of biofuels such as bioethanol and biomethane from corn grain and sugarcane and biodiesel from soybean and rapeseed at an economically viable scale. However, these biocrops did not fit the requirements of reduction of greenhouse gas emissions because of the high energy inputs, such as for nitrogen (N) fertilization (Hill, 2007; Tilman et al., 2009). Additionally, using farm land to produce biofuel could lead to a food security issue. Therefore, in order to avoid competition with food crops, second-generation biofuels are produced with lignocellulosic biomass available from organic waste, and agricultural and forestry residues. Moreover, the biomass production from the second-generation energy crops should be high-yielding with low inputs of water, fertilizers and energy and should be grown on marginal lands to avoid competition with food crops (F. Anderson et al., 2008; Quinn et al., 2015). Perennial biomass crops can fulfill the demands for biomass production because they are more stress tolerant than annual crops and the root system can store nutrition for coming years. Thanks to the well-developed root system of perennial plants, they can adapt to poor soils and do not require large amounts of fertilizer. Moreover, development of deep roots helps to overcome drought stress. One of the most promising perennial crops for bioenergy production is *Miscanthus*. Stress tolerant genotypes have been identified in various *Miscanthus sinensis* and *Miscanthus sacchariflorus* genotypes (Lewandowski et al., 2016). These indeed have the potential to be grown on marginal lands and store nutrition in the roots and rhizomes during the winter. Besides, the productive period of this perennial biomass crop ranges from 10 to 25 years without annual ploughing, which prevents soil erosion, sequestrates soil carbon and improves agricultural ecosystems (Lewandowski et al., 2000; McCalmont et al., 2017). Perennial grasses such as switchgrass or *Miscanthus* with a C4 photosynthetic pathway have better water and nutrient use efficiency and increase the land use efficiency as well.

1.1 Biofuel production

Currently, C4 crop-derived perennial biomass is utilized mostly for direct combustion, or for anaerobic digestion to produce biogas (Sanscartier et al., 2014; Jeswani et al., 2015; Kiesel et al., 2017). Compared to fossil fuels, bioenergy crops use photosynthesis to re-fixate carbon dioxide that was emitted during combustion of biogas or biomass (Wagner and Lewandowski, 2017). Because the quality and needs for either combustion or biogas are different, the effect of the marginal conditions on energy production from grown on these soils, and on the composition of biomass needs to be considered. For combustion, the most cost-effective way of energy production is to burn the chips that are cut from *Miscanthus* directly, and high quality material is demanded with low moisture, low minerals and low ash content (Smith and Slater, 2011; Baxter et al., 2012; Iqbal et al., 2017). High moisture content in biomass not only increases the amount of combustion gases but also reduces the calorific value. High mineral content in the biomass causes low melting temperatures and corrosive issues for the boilers. High ash content also reduces the energy output. Optimal heat transfer form biomass requires low moisture and mineral content. For this purpose, the raw biomass must be harvested at minimum moisture and mineral content, i.e. from late autumn to early spring (Lewandowski et al., 2003b; Wagner et al., 2017).

Biogas produced by anaerobic digestion can be stored in the existing natural gas infrastructure and further utilized as a transport fuel. Compared with first-generation biofuel generated mainly from starch, the recalcitrance of lignocellulose to hydrolysis adds an additional level of complexity (McCann and Carpita, 2015). Converting the biomass to biogas takes three major steps: (1) pretreatment of the biomass feedstock; (2) hydrolysis and saccharification; (3) fermentation of sugars into ethanol or methanol. Cell wall composition is the most important factor for the convertion efficiency from biomass to biogas. Thus, easy digestible biomass (consisting of high hemicellulose but low lignin) is considered as the best quality for producing biogas. For instance, high lignin content negatively affects the efficiency of cell wall polysaccharide digestion into fermentable sugars (Chen and Dixon, 2007; Himmel and Picataggio, 2009; Studer et al., 2011). Lignin linked to cellulose and hemicellulose inhibits the release of polysaccharides during the pretreatment process or reduces the accessibility of enzymes during the conversion process (Palmqvist and Hahn-Hägerdal, 2000; López et al., 2004). High lignin content of biomass was shown to have negative effects on the saccharification efficiency of *Miscanthus* (van der Weijde et al., 2016).

1.2 The crop *Miscanthus*

Miscanthus, a C4 grass genus, is native to eastern and southeastern Asia and the south Pacific islands with the highest species diversity in China and Japan (Sun et al., 2010). Its natural distribution ranges from temperate areas to tropical regions and from mountains to coastal areas (Hodkinson et al., 2014). The high diversity and adaption to different areas supports a diverse germplasm for breeding programs aiming at varieties that can grow on marginal lands (Jones et al., 2015). The genus *Miscanthus* has a basic chromosome number of 19 with polyploidy features, including diploids, triploids, tetraploids, pentaploids, and hexaploids (Hodkinson et al., 2001; Hodkinson et al., 2002a; Hodkinson et al., 2002b). A sterile hybrid, *Miscanthus × giganteus* (*M. sacchariflorus × M. sinensis*), with high yield and quality has been widely researched and used as biofuel crop in Europe (Lewandowski et al., 2003a). This triploid interspecific hybrid (3n =3x =57) can be propagated by rhizomes or tissue culture but this requires higher plantation costs than seeds (Greef and Deuter, 1993; Xue et al., 2015). In addition, improving the genetics by direct crossing with this genotype is impossible because it is sterile. The diploid species *M. sinensis* (2n =2x =38) and the tetraploid species *M. sacchariflorus* (2n =4x =76), which are the parents of *Miscanthus* × *giganteus*, can be crossed and used for breeding new genotypes and improved interspecific hybrids. A significant concern for *Miscanthus* as a nonnative perennial with broad environmental adaptation is that it may become an invasive weed (Barney and DiTomaso, 2008). Sterile hybrids such as *Miscanthus* × *giganteus* can reduce the risk of invasion in the field conditions. Interspecific hybrids, including *Miscanthus* × *giganteus* showed higher yield than *M. sinensis* and *M. sacchariflorus* genotypes in six field trials across Europe (Kalinina et al., 2017). Compared with *M. sacchariflorus*, *M. sinensis* not only produces seeds easily but also regenerates from callus after whole genome duplication (Chae et al., 2013). Thus, a good alternative to the *M. sinensis* × *M. sacchariflorus* hybrid is to produce hybrid seeds by crossing diploid *M. sinensis* (2n =2x =38) and tetraploid *M. sinensis* (2n =4x =76) (J. Rounsaville et al., 2011). To meet the commercial production needs, sexual hybridization not only improves germplasm but also reduces plantation costs by using seeds and seedlings rather than propagation from rhizomes or tissue (Hastings et al., 2017). There are two types of seed-based propagation for commercial production. Hybrid seeds can be produced by pairwise crosses, or synthetic cultivars can be generated by intercrossing a number of genotypes (Sacks et al., 2013). For pairwise crosses, two genotypes that flower concurrently need to be put together that can produce seeds from known male and female parents, while synthetic cultivars generate mixtures of seeds from superior performing parental genotypes by open pollination. At Wageningen University & Research the *Miscanthus* breeding program explores the potential of *M. sinensis* for seed production and commercial cultivation. In addition, molecular tools are developed for to accelerate breeding: a genetic map was created for a mapping population segregating for quality traits and used to dissect genetic variation for a variety of traits related to cell-wall properties (van der Weijde et al., 2017).

1.3 The advantages of *Miscanthus*

Numerous studies have evaluated different species as bioenergy crops and *Miscanthus* × *giganteus* has been considered as a high potential feedstock because of several specific properties (Kiesel et al., 2017). Firstly, *Miscanthus* is a C4 plant and has a more efficient photosynthesis than C3 plants due to the ability to be particularly effective in utilizing CO_2. Secondly, the biomass of *Miscanthus* for combustion has higher quality than wheat straw. Post-senescence harvests allow nitrogen and other minerals to be translocated from the shoots into the rhizomes prior to harvest, and the dead biomass is low in moisture content. This nutrient cycling not only reduces fertilizer requirements but also improves the combustion quality because of lower mineral content, which reduces corrosiveness (Jorgensen, 1997; Smith and Slater, 2011; Jensen et al., 2017). Thirdly, *Miscanthus* sequesters carbon to below-ground biomass and increases organic matter of the soil through dropping of senesced leaves before harvest and because of old root decomposition, which improves soil fertility (Kuzyakov and Domanski, 2000). Finally, as already mentioned, there is rich genetic variation in wild populations of *Miscanthus* (Hodkinson et al., 2014) and the high diversity of *Miscanthus* provides a valuable breeding resource for varieties that can be grown on marginal lands. Global climate change leads to increased marginalization of agricultural lands. For example, higher temperatures increase the effects of droughts, and drought and poor irrigation under high evaporative conditions leads to salinization of the soil. A rise in sea level causes additional accumulation of salt in coastal regions. Since *Miscanthus* species shows moderate tolerance to drought, salt, flooding, and chilling, it is possible that this bioenergy crop can support a viable economical biomass even under marginal conditions.

1.4 Breeding target

Development of tolerant genotypes that can withstand different stressful environments is an important step to expand *Miscanthus* cultivation to marginal lands (Lewandowski et al., 2016). Not only total yield but also quality of the biomass should be taken into account. High quality materials not only allow efficient bioenergy production but also decrease the maintenance costs for combustion equipment. Breeding in *Miscanthus* basically should focus on producing genotypes that can provide high yield and quality biomass on marginal lands. Several quality traits for combustion are important, such as low ash contents and moisture and low levels of minerals such as Cl^- and K^+ to minimize corrosive issues. Quality traits for enhancing saccharification efficiency include high ratios of hemicellulose but low cellulose and lignin to improve digestibility (Himken et al., 1997). In addition to yield and quality, there are several other factors that need to be considered carefully. Because *Miscanthus* originates from Asia, invasiveness into the ecosystem in Europe needs to be taken into account. *Miscanthus* × *giganteus*' sterility is an advantage with respect to invasiveness. To reduce the risk of spread into natural habitats, novel sterile genotypes (triploid) were crossed from fertile parent species (tetraploid *M. sacchariflorus* and diploid *M. sinensis*), producing high-yielding hybrids (Kalinina et al., 2017). However, these sterile genotypes need to be propagated by tissue culture or rhizomes, which increases plantation cost. When *Miscanthus* is grown in native regions without invasive issues, sowing seeds simply decreases the cost of plantation compared with seedlings from rhizomes or tissue culture (Hastings et al., 2017). It is estimated that the cheapest way to plant *Miscanthus* could be direct seed sowing (Xue et al., 2015). It is also possible to use fertile *M. sinensis* or *M. sacchariflorus* to improve the genetics and develop novel genotypes. Especially, the pollen fertility rate of *M. sinensis* is more than 86% and the self-pollination rate is very low (Yamada, 2015). However, the yield of these genotypes is typically lower than that of the interspecific hybrids. To upscale the *Miscanthus* crop to millions of hectares and reduce plantation cost, using hybrid seeds in modular plugs for precommercial scale trials have been set up. The results of these experiments will produce much needed information of the performance of new hybrids tested in different locations (Clifton-Brown et al., 2017).

2 Abiotic stress with emphasis on salt stress

Marginal lands limit plant growth and yield due to poor soils, low water availability, low nutrient, high salinity and presence of toxic elements. Climate change increases abiotic stresses such as drought, temperature, and salinity. Drought stress negatively affects yield, inducing growth reduction and early senescence. Due to unbalance of water potential in plants under drought, a loss of turgor changes the cell wall composition and reduces cell size (Tenhaken, 2015). Extremely low temperature can be lethal for plants so the winter mortality of plantations should be considered in high latitude regions (Clifton-Brown and Lewandowski, 2000). Frost may kill the rhizomes during winter and damage newly emerged shoots in spring. Salt stress is considered one of the most severe abiotic stresses for plants. Soil salinity is a global issue for agricultural productivity and almost 20% of the world's irrigated lands is affected by salinity (Flowers and Yeo, 1995; Munns and Tester, 2008; Rengasamy, 2010; Qadir et al., 2014; Butcher et al., 2016). It is even predicted that 50% of irrigated land will be affected by salinity in 2050 due to poor drainage and climatic change (Bartels and Sunkar, 2005). Saline soils are classified by solute electrical conductivity (EC) in deciSiemens/meter (dS/m) as: nonsaline (<2 dS/m, approximately equal to 20 mM NaCl), slightly saline (2-4 dS/m, approximately equal to 20-40 mM NaCl), moderately saline (4-8 dS/m, approximately equal to 40-90 mM NaCl), strongly saline (8-16 dS/m, approximately equal to 90-170 mM NaCl), and very strongly saline (>16 dS/m, approximately equal to 170 mM NaCl) (Rhoades, 1999). Under field conditions, salt stress persistence may co-occur with additional stresses such as other abiotic factors (e.g. heat, drought, flooding, etc.) and biotic factors (fungi, bacteria, insects, etc.). This leads to a more complex and multi-stress environment for plants. For example, flooding may also cause a dramatic change in EC value, and heat may increase the evaporation rate which also increases the salt stress. The strong effect of salinity on crop yield and quality makes salinity tolerance in crops an important target for breeding. Genetic improvement of crops is one of the most important strategies to improve the yield and quality under salt stress. However, breeding for salt tolerance is not straightforward due to its genetic complexity.

2.1 Response of plants under salt stress

Salinity seriously reduces plant growth and changes its metabolism, because salt stress includes osmotic stress, ion toxicity stress, and nutritional imbalance (Ashraf and Harris, 2004; Munns and Tester, 2008). Osmotic stress due to the high concentration of ions in the root environment induces stomatal closure, reducing the transpiration and growth rate (Munné-Bosch et al., 2001). Individual cells also become smaller and thinner under osmotic stress compared to normal conditions (Iraki et al., 1989; Le Gall et al., 2015). These responses of plants to salinity show similarities to the response under drought stress. Ion toxicity stress develops over time because the ions accumulate to toxic levels within the shoots. As NaCl is a major constituent of saline soil, both Na^+ and Cl^- can accumulate to toxic levels in the plant. Na^+ and Cl^- are both toxic to plants at high concentrations but some species can control Na^+ transport better than Cl^- and vice versa (Munns and Tester, 2008; Teakle and Tyerman, 2010). For example, genotypes of *Glycine max* were more sensitive to Cl^- accumulation, but *Glycine soja* genotypes were more sensitive to high levels of Na^+ ions (Luo et al., 2005). There have been numerous reviews on the mechanisms of Na^+ transport in plants under salt stress (Blumwald et al., 2000; Garciadeblas et al., 2003; Sánchez-Aguayo et al., 2004; Huang et al., 2006). The high Na^+ concentrations not only interfere with K^+ uptake and K^+ function but also inhibit metabolic pathways (Shabala and Cuin, 2008) so a high ratio of K^+/Na^+ in the leaves is considered as a salt tolerance trait (Maathuis and Amtmann, 1999; Munns and James, 2003). Bread wheat maintains a high ratio of K^+/Na^+ in the leaves, which contributes to salt tolerance, while durum wheat is more salt-sensitive due to a low ratio of K^+/Na^+ (Gorham et al., 1990). The degree of senescence in mature leaves reflects ion-specific toxicity due to high levels of Na^+ concentrations without tissue tolerance to Na^+ (Munns and James, 2003). In contrast to Na^+ (see below), the mechanisms of Cl^- transport are less well understood (Teakle and Tyerman, 2010). Although Cl^- is considered an essential micronutrient involving in photosynthesis, turgor and pH regulation (Teodoro et al., 1998; White and Broadley, 2001), high levels of Cl^- accumulation in the shoots are toxic for Cl^- sensitive species (4-7 mg/g dry weight) and for Cl^- tolerant species (15-50 mg/g dry weight) (Xu et al., 1999), and symptoms of chloride toxicity in plants include necrosis of leaf margins and tips. Several studies demonstrated that regulation of Cl^- homeostasis may be important for salt tolerance in some crops (Tavakkoli et al., 2010; Teakle and Tyerman, 2010).

2.2 Mechanisms of salt tolerance in plants

Plants can utilize different mechanisms to reduce damage and deal with the osmotic stress and ionic stress components of salt stress (Munns and Tester, 2008). An overview of responses to salt stress in plants was shown in Figure 1. There are three common salt tolerance mechanisms in plants (Munns and Tester, 2008; Rajendran et al., 2009):

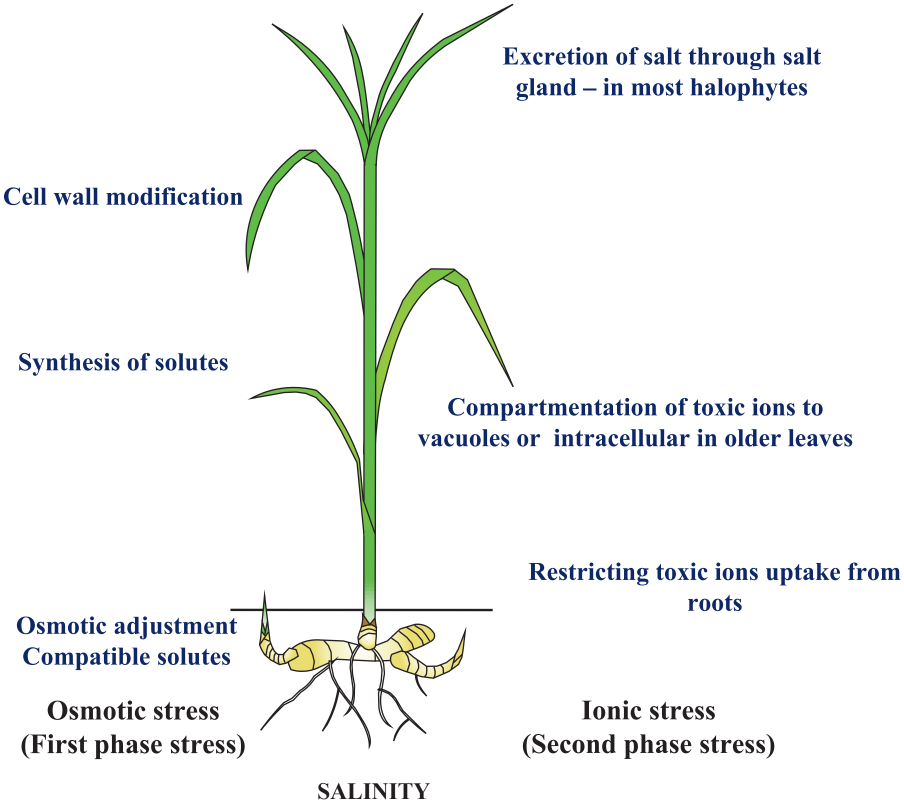

Figure 1. An overview of plant adaptive responses to salt stress. Salt stress causes both osmotic stress (first phase stress) and ionic stress (second phase stress). Osmotic stress leads to water deficit and dehydration and plants synthesize compatible solutes to regulate cell turgor. Rhizome tissues storing starch can produce soluble sugars as compatible solutes or osmotic adjustments to deal with osmotic stress. Cell walls can reduce size and thickness to limit growth and maintain turgor. High concentrations of Na^+ and Cl^- in leaves cause ionic stress and are toxic to plant cells. Glycophytes adapt to ionic stress by restricting ion accumulation in roots and leaves and use ion exclusion mechanisms to avoid ion accumulation in the shoots. Ion sequestration into vacuoles and depositing salt in older leaves helps to protect young, growing tissues. Halophytes can also utilize salt glands to secrete ions.

(1) Osmotic tolerance

The osmotic stress directly lessens cell growth both in root tips and leaves, and causes stomatal closure. Stomatal closure diminishes water loss through transpiration. Under osmotic stress, maintaining the cell turgor can be achieved by adapting root water uptake properties, plant hydraulics, and by adjusting the plant's osmotic potential by producing compatible solutes. Compatible solutes are small water-soluble molecules such as proline (Khatkar and Kuhad, 2000), glycine betaine (Khan et al., 2000; Wang and Nii, 2000), sugars (Kerepesi and Galiba, 2000), and polyols (Bohnert et al., 1995; Zhifang and Loescher, 2003). These compatible solutes not only regulate the osmotic balance but also protect cellular structures, for instance by scavenging Reactive Oxygen Species (ROS) (Hasegawa et al., 2000).

(2) Na^+ exclusion mechanism

Since high concentrations of Na^+ and Cl^- are toxic to plant cells, avoiding toxic levels of these ions is an important determinant of salt tolerance. Maintaining a high ratio of K^+/Na^+ in the leaves contributes to salt tolerance because of the competition between K^+ and Na^+. Several transporters have been identified and characterized in plants that regulate the balance of Na^+ and the K^+/Na^+ ratio. The HKT transporter (High-affinity K^+ Transporters) family is composed of two subfamilies. Gene members of subfamily 1 are all Na^+-specific transporters, but gene members of subfamily 2 are either Na^+-K^+ co-transporters or Na^+ and K^+ uni-porters (Horie et al., 2001; Maser et al., 2002; Garciadeblas et al., 2003; Almeida et al., 2013). Members of the HKT transporter family play important roles in shoot Na^+ exclusion (Conde et al., 2011; Munns et al., 2012; Platten et al., 2013; Wang et al., 2014). For instance, shoot exclusion was shown to be facilitated by HKT1;5, which can take Na^+ from the xylem into the parenchyma cells and minimize the accumulation of Na^+ in the shoot (Conde et al., 2011). The leaf Na^+ concentration in the leaves was shown to be highly associated with *HKT1;5* allelic variation across rice diverse accessions (Platten et al., 2013).

(3) Tissue tolerance

An inclusion mechanism which compartmentalizes Na^+ and Cl^- in cellular and intracellular organelles like the vacuoles (Adams et al., 1992), thus protecting cytosolic processes from the effects of high ion concentrations, is often utilized by halophytes. Members of the NHX (Na^+/H^+ antiporter) gene family sequester Na^+ in the vacuoles and maintain osmotic balance (Apse et al., 1999; Glenn et al., 1999). Similarly, anion transporters such as CLC transport Cl^- into the vacuoles (Teakle and Tyerman, 2010). There are three types of NHX in most plants, located in the plasma membrane, the vacuolar membrane, or endosomal membranes (Bassil et al., 2012). Tonoplastic NHX proteins are involved in regulation of ion concentrations as well as cytosolic pH value (Bassil et al., 2012). In the glycophyte maize, *ZmNHX*1 and *ZmNHX*6 were highly expressed in the root to maintain relatively high ion concentrations in roots but low ion contents in shoots in salt tolerant genotypes (Zörb et al., 2005).

2.3 Response of *Miscanthus* to salt stress

Only few studies have been done in relation to salt tolerance of *Miscanthus* and most of these focused on *Miscanthus* × *giganteus* (Plazek et al., 2014; Sun et al., 2014; Stavridou et al., 2016). Stavridou et al. (2006) found that there was no significant reduction in shoot dry weight at very mild saline conditions, up to 60 mM NaCl. Shoot dry weight was significantly decreased at 90 mM NaCl and 50% reduced at 120 mM NaCl. Therefore, *Miscanthus* × *giganteus* may be considered a moderately salt tolerant plant (Quinn et al., 2015). When the salinity was higher than 120 mM NaCl, proline contents, ash contents and Na^+ and Cl^- in the leaves significantly increased compared with control conditions (Plazek et al., 2014; Stavridou et al., 2016), indicating that a salt stress of 120 mM NaCl affects both growth and ion homeostasis of *Miscanthus* (Plazek et al., 2014; Stavridou et al., 2016). With respect to the combustion quality, high ash contents and ion contents in biomass can be harmful to combustion equipment because high ash contents reduce the energy output derived from biomass (James et al., 2012), and high ion contents causes corrosion issues (Brosse et al., 2012). Until now the genetic diversity for salt tolerance in *Miscanthus* germplasm has not been explored, although *M. sinensis* var. Condensatus can be cultivated on seashores (Chou et al., 1999) and Sun et al. (2014) indicated that wild *M. sinensis* in Japan may harbor significant genetic variation for salt tolerance. Thus, evaluating the salt tolerance diversity in *Miscanthus* is likely to identify valuable sources for breeding programs aimed at improved salt tolerant cultivars with high biomass quality even when cultivated under saline conditions. This would enable expansion of the cultivation to saline areas, avoiding competition with food crops for arable lands while still achieving economically viable yields.

3 This thesis

In this thesis, the genetic diversity of *Miscanthus* for salt tolerance, and the effects of salt stress on the biomass quantity and quality in *Miscanthus* germplasm were studied.

The aims were:

To explore the genetic diversity of *Miscanthus* germplasm for salt tolerance;

To identify *Miscanthus* genotypes that can be grown on saline soils;

To identify *Miscanthus* genotypes that can serve as starting material for breeding for improvement of salt tolerance;

To investigate the impact of salinity on biomass quality for combustion or anaerobic digestion.

In chapter 2, a collection of genotypes with different genetic backgrounds, including *M. sacchariflorus*, *M. sinensis*, and the triploid interspecific hybrid *Miscanthus* × *giganteus,* was evaluated on a hydroponic growth system. Traits that may contribute to salt tolerance were measured and highly salt tolerant and high yielding genotypes under salt stress were identified as potential breeding materials. In addition, tolerant genotypes were found to employ different mechanisms to withstand salt stress.

In chapter 3, twelve genotypes with either high salt tolerance or high yield on the hydroponic system were analyzed in-depth at different salt levels in pots under greenhouse conditions. In addition, a comparison was made between plants grown from rhizomes or from *in vitro* cuttings propagated on hydroponics. At 250 mM NaCl, rhizome- as well as *in vitro*-derived genotype OPM-56 demonstrated a relatively high yield and salt tolerance and low ion contents, which is a good characteristic for combustion. This genotype with a strong ion exclusion mechanism can be considered as a candidate for improving salt tolerance in *Miscanthus* varieties.

Although several salt tolerant genotypes have been identified, the effects of salt stress on the quality of biomass are still largely unknown. Therefore, Chapter 4 explores the implications of salt stress on cell wall quality of *Miscanthus* biomass for anaerobic digestion. The cell wall compositions of plants grown from *in vitro*-propagated plantlets in pots outdoors under a roof were studied. Plants were allowed to finish their growth cycle and harvested in winter. The cell wall composition was shown to be affected by salinity, and these changes are likely to affect anaerobic digestion.

Chapter 5 describes the evaluation of *M. sacchariflorus* accessions collected from different areas in China in a two-year field trial on saline marginal lands. Under low-input and salinity conditions, the accessions originating from the sea coast were found to have the highest salt stress tolerance and yield. Using these local accessions as salt tolerant resources can accelerate the breeding programs for salt tolerant *Miscanthus* varieties.

The general discussion in chapter 6 discusses the results in this thesis in relation to the current status and the aims of breeding for salt tolerance in *Miscanthus*. The salt tolerant genotypes identified by different screening strategies can contribute to the genetic improvement of *Miscanthus* as a lignocellulosic feedstock and enable expansion of this bioenergy crop to marginal lands.

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Chapter 2

Genetic diversity of salt tolerance in *Miscanthus*

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Keywords: *Miscanthus*, salt tolerance, osmotic stress, ionic stress, ion homeostasis.

Published in Fronties of Science

Chen, C.-L., van der Schoot, H., Dehghan, S., Alvim Kamei, C.L., Schwarz, K.-U., Meyer, H., Visser, R.G.F., and van der Linden, C.G. (2017). Genetic Diversity of Salt Tolerance in *Miscanthus*. Frontiers in Plant Science 8: 187.

Abstract

Miscanthus is a woody rhizomatous C4 grass that can be used as a CO_2 neutral biofuel resource. It has potential to grow in marginal areas such as saline soils, avoiding competition for arable lands with food crops. This study explored genetic diversity for salt tolerance in *Miscanthus* and discovered mechanisms and traits that can be used to improve the yield under salt stress. Seventy genotypes of *Miscanthus* (including 57 *M. sinensis,* 5 *M. sacchariflorus* and 8 hybrids) were evaluated for salt tolerance under saline (150 mM NaCl) and normal growing conditions using a hydroponic system. Analyses of shoot growth traits and ion concentrations revealed the existence of large variation for salt tolerance in the genotypes. We identified genotypes with potential for high biomass production both under control and saline conditions that may be utilized for growth under marginal, saline conditions. Several relatively salt tolerant genotypes had clearly lower Na^+ concentrations and showed relatively high K^+/Na^+ ratios in the shoots under salt stress, indicating that a Na^+ exclusion mechanism was utilized to prevent Na^+ accumulation in the leaves. Other genotypes showed limited reduction in leaf expansion and growth rate under saline conditions, which may be indicative of osmotic stress tolerance. The genotypes demonstrating potentially different salt tolerance mechanisms can serve as starting materials for breeding programs aimed at improving salinity tolerance of *Miscanthus*.

1 Introduction

Miscanthus is a C4 perennial grass originating from Southeast Asia, the Pacific islands, and tropical Africa. The genus *Miscanthus* has a basic chromosome number of 19 and includes the nominally diploid species *Miscanthus sinensis* (2n =2x =38) and tetraploid species *Miscanthus sacchariflorus* (2n =4x =76) plus a triploid interspecific hybrid, *Miscanthus* × *giganteus* (3n =3x =57). This hybrid was identified as a good candidate for energy production by direct combustion (Zub and Brancourt-Hulmel, 2010). However, *Miscanthus* × *giganteus* has several disadvantages. Since *Miscanthus* × *giganteus* is a sterile triploid, it is difficult to improve its genetics by crossing. In addition, its sterility requires propagation from rhizomes or tissue culture, which is relatively more expensive than from seeds (Greef and Deuter, 1993). To screen and explore natural genetic diversity from other sources is therefore important for genetic improvement of the crop. A good alternative for breeding purposes is the diploid species *M. sinensis*. An important breeding goal for any bioenergy crop and also *Miscanthus* is to achieve economically viable yields in marginal lands, thus avoiding competition with food crops and interfering with food security (Somerville et al., 2010).

High soil salinity is one of the major constraints of crop growth because it decreases crop yield and quality. Almost 20% of the world's irrigated land is adversely influenced by salinity (Flowers and Yeo, 1995; Munns and Tester, 2008; Rengasamy, 2010b; Qadir et al., 2014), and the problem of soil salinity is further increasing because of poor drainage and climatic change (Bennett and Khush, 2003). Salinity affects plant growth because of osmotic stress, ionic stress, and nutritional imbalance (Ashraf and Harris, 2004; Munns and Tester, 2008). Osmotic stress affects growth immediately and is in saline soils caused by limitation of water uptake resulting from the high salt concentration in the soil. Ionic stress develops over time and is due to ion accumulation within the shoots. Osmotic stress accounts for roughly 75% of the biomass decrease under salt stress, and ionic stress reduces it by another 20% (Munns and Tester, 2008). The strong effect of salinity on crop yield makes salinity tolerance in crops an important target for breeding. However, breeding for salt tolerance is not straightforward due to its genetic complexity.

Salt stress affects all the major processes underlying plant growth, including lipid and energy metabolism, photosynthesis, and protein synthesis (Parida and Das, 2005). This leads to reduction in transpiration, chlorophyll content, tiller number, and biomass (Hassanein, 1999; Chartzoulakis and Klapaki, 2000). The altered water status and unbalanced ion homeostasis resulting from saline conditions induce several mechanisms to reduce damage in the plant. Osmotic tolerance can be achieved by adapting water uptake properties of the roots, plant hydraulics, and by adjusting the plant’s osmotic potential. Production of compatible solutes like proline (Khatkar and Kuhad, 2000), glycine betaine (Khan et al., 2000; Wang and Nii, 2000), sugars (Kerepesi and Galiba, 2000), and polyols (Bohnert et al., 1995; Zhifang and Loescher, 2003) facilitate osmotic adjustment or osmotic protection. To avoid toxic ion concentrations in shoots, plants exclude exccess sodium and chloride ions from the shoot. Bread wheat for instance has a low rate of Na^+ transport to the shoot and maintains a high ratio of K^+/Na^+ in the leaves, which contributes to salt tolerance, while durum wheat is more salt-sensitive due to its poor ability to exclude Na^+ from the shoot (Gorham et al., 1990). Shoot exclusion was shown to be facilitated by a members of the high-affinity K^+ transporter (HKT) family (HKT1;5) that can take Na^+ from the xylem into the parenchyma cells to minimize the accumulation of Na in the shoot (Conde et al., 2011). Tissue tolerance to high salt concentrations is a mechanism often utilized by halophytes, and it can be achieved by compartmentalization of Na^+ and Cl^- in cellular organelles like the vacuoles (Adams et al., 1992) and involves tonoplast Na^+/H^+ antiporters (NHX) that regulate cytosolic Na^+ concentration and pH (Bassil et al., 2012). In mature leaves, senescence may reflect the toxic effect of high levels of Na^+ concentration and low tissue tolerance to Na^+ (Munns and James, 2003). The combination of accumulation of Na^+ in leaves, lack of necrosis, and relatively little reduction of biomass can be indicative of tissue tolerance (Munns and James, 2003; Rajendran et al., 2009).

Salt stress not only affects the quantity but also the quality of *Miscanthus* biomass. *Miscanthus* genotypes with less ions in the harvestable biomass are particularly important because high concentrations of minerals can be corrosive to combustion equipment (Jorgensen, 1997). Thus, it is essential for *Miscanthus* to produce stable biomass with low ion concentrations under salt stress. Only few studies have been done in relation to salt tolerance of *Miscanthus* (Li et al., 2014; Sun et al., 2014; Plazek et al, 2014; Stavridou, 2016), and *Miscanthus* may be considered a moderately salt tolerant crop with salt concentrations higher than 100 mM NaCl (approximately 10 dS/m) reducing crop yields considerably. Until now the genetic diversity of salt tolerance in *Miscanthus* germplasm has not been investigated, although Sun et al. (2014) indicate that *M. sinensis* may harbour significant genetic variation for salt tolerance. The current study aims to explore genetic diversity of *Miscanthus* breeding materials to identify genotypes for cultivation in saline soils, and genotypes that harbor salt tolerance traits and can serve as materials for improvement of *Miscanthus* salt tolerance. The results showed that several genotypes with relatively high salt tolerance appeared to rely on different mechanisms, offering opportunities for breeding programs aimed at improving tolerance of *Miscanthus*.

2 Materials and Methods

2.1 Plant materials

Seventy genotypes of *Miscanthus* were evaluated for salt tolerance (Table 1). The set included 57 *M. sinensis*, 5 *M. sacchariflorus* and 8 hybrids (OPM-9 is *Miscanthus* × *giganteus*) and each genotype was cloned and propagated by tissue culture. The genotypes were supplied by different sources (Aberystwyth University, Institute for Agricultural and Fisheries Research ILVO, and Wageningen University & Research). Two genotypes were tested in a pilot experiment to establish optimal experimental conditions.

2.2 Pilot experiment

Two genotypes (OPM-13 and OPM-38) were grown under different levels of salinity (0 mM, 125 mM, and 250 mM NaCl). The seedlings were propagated *in vitro*, transferred to the hydroponics system and allowed to acclimate for 1 week. The hydroponics system consisted of containers (22 L, 40 cm length, 30 cm width and 20 cm height) that can hold up to 12 *Miscanthus* plants. A maximum of 16 containers can be connected as a unit to a single reservoir, with capacity of 500L nutrient solution. For the pilot experiment, three units were used for the three different salt levels, each with 2 connected containers. The nutrient solution was half-strength modified Hoagland's solution (Supplemental Table 1), maintained at pH 5.8 and refreshed weekly. Seedlings with four leaves were selected and transferred to the hydroponics containers. Each container had two genotypes in four replications (8 plants). After one week of acclimation, NaCl was added to the nutrient solutions of two of the units with a 25 mM daily increment until a concentration of 125 mM NaCl. Only one of those units received two more additions of 62.5 mM NaCl to reach 250 mM NaCl. The average day/night temperatures were set at 25/18 °C, and the photoperiod regime was 16 hours light and 8 hours dark. Greenhouse environmental humidity was controlled at 70%. Additional lighting (100 Wm^{-2}) was used when the incoming shortwave radiation was below 200 Wm^{-2}. After 2 weeks of salt treatment the shoot dry weight and Na^+ and Cl^- concentrations of the shoots were measured and evaluated.

Table 1. *Miscanthus* genotypes screened for salt tolerance. The OPM code for the genotypes was used within the EU project OPTIMISC.

No.	Supplier	Genotype	No.	Supplier	Genotype
OPM-4	IBERS	*M. sacchariflorus*	OPM-66	WUR	*M. sinensis*
OPM-5	IBERS	Hybrid (*M. sinensis* × *M. sacchariflorus*)	OPM-67	WUR	*M. sinensis*
OPM-6	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)	OPM-68	WUR	*M. sinensis*
OPM-7	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)	OPM-69	WUR	*M. sinensis*
OPM-8	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)	OPM-71	WUR	*M. sinensis*
OPM-9	IBERS	Hybrid (*Miscanthus* × *giganteus*)	OPM-72	WUR	*M. sinensis*
OPM-10	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)	OPM-73	WUR	*M. sinensis*
OPM-11	IBERS	*M. sinensis*	OPM-74	WUR	*M. sinensis*
OPM-13*	WUR	*M. sinensis*	OPM-75	WUR	*M. sinensis*
OPM-16	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)	OPM-76	WUR	*M. sinensis*
OPM-19	IBERS	*M. sacchariflorus*	OPM-77	WUR	*M. sinensis*
OPM-20	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)	OPM-78	WUR	*M. sinensis*
OPM-24	IBERS	*M. sacchariflorus*	OPM-79	WUR	*M. sinensis*
OPM-26	IBERS	*M. sacchariflorus*	OPM-81	IBERS	*M. sinensis*
OPM-30	IBERS	*M. sinensis*	OPM-82	WUR	*M. sinensis*
OPM-31	IBERS	*M. sinensis*	OPM-83	WUR	*M. sinensis*
OPM-32	IBERS	*M. sinensis*	OPM-84	WUR	*M. sinensis*
OPM-33	IBERS	*M. sinensis*	OPM-86	WUR	*M. sinensis*
OPM-34	IBERS	*M. sacchariflorus*	OPM-87	WUR	*M. sinensis*
OPM-37	WUR	*M. sinensis*	OPM-88	WUR	*M. sinensis*
OPM-38*	WUR	*M. sinensis*	OPM-89	WUR	*M. sinensis*

(Continued)

Table 1. Continued

No.	Supplier	Genotype	No.	Supplier	Genotype
OPM-41	WUR	*M. sinensis*	OPM-90	WUR	*M. sinensis*
OPM-42	WUR	*M. sinensis*	OPM-91	WUR	*M. sinensis*
OPM-44	WUR	*M. sinensis*	OPM-92	WUR	*M. sinensis*
OPM-45	WUR	*M. sinensis*	OPM-94	WUR	*M. sinensis*
OPM-47	WUR	*M. sinensis*	OPM-96	IBERS	*M. sinensis*
OPM-48	WUR	*M. sinensis*	OPM-97	IBERS	*M. sinensis*
OPM-49	WUR	*M. sinensis*	OPM-98	WUR	*M. sinensis*
OPM-50	WUR	*M. sinensis*	OPM-99	WUR	*M. sinensis*
OPM-56	WUR	*M. sinensis*	OPM-100	ILVO	*M. sinensis*
OPM-57	WUR	*M. sinensis*	OPM-101	WUR	*M. sinensis*
OPM-58	WUR	*M. sinensis*	OPM-103	WUR	*M. sinensis*
OPM-59	WUR	*M. sinensis*	OPM-104	WUR	*M. sinensis*
OPM-62	WUR	*M. sinensis*	OPM-107	WUR	*M. sinensis*
OPM-64	WUR	*M. sinensis*	OPM-108	WUR	*M. sinensis*
OPM-65	WUR	*M. sinensis*	OPM-109	IBERS	*M. sinensis*

(IBERS) Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, UK
(ILVO) The Institute for Agricultural and Fisheries Research, Belgium
(WUR) Wageningen University & Research, the Netherlands
* In pilot experiment

2.3 Main experiment design

Seedlings from the 70 genotypes were propagated *in vitro* for 6 weeks and allowed to form roots. Then they were transferred to the greenhouse and allowed to acclimate for 2 weeks on hydroponic containers in the greenhouse (Unifarm, Wageningen University & Research). Uniform seedlings with four leaves were selected and transferred to the hydroponics system for evaluation. Four independently controlled hydroponics units were used; two units for control and the other two for the salt treatment (Supplemental Figure 1), and each unit consisted of twelve connected containers that could hold 12 plants. The hydroponics system was filled with half-strength modified Hoagland's solution. After 1 week in the hydroponics system, NaCl was added to two of the four units with a 50 mM daily increment to bring the final concentration to 150 mM NaCl. The experiment had a split plot design with four replicate plants per genotype per treatment. For this, the 70 genotypes and 2 dummy plants were randomly assigned to the plant positions in 6 containers as one replication. Two replications of 70 genotypes were grown in 12 containers on each unit, to a total of 4 replications on two units per treatment. The nutrient solution was refreshed weekly and maintained at pH 5.8. The greenhouse conditions were similar to the pilot experiment.

2.4 Assessment of growth traits

During the experiment, data was collected for plant height, leaf expansion, and tiller number for all plants grown under control and saline conditions. Plant height was measured from the base of the plant to the tip of the highest leaf with a ruler at day 1, day 10 and day 17 after starting the stress treatment. Growth rate was taken as the growth in height per day, expressed as cm/day. This was calculated as the difference in plant height between two timepoints, divided by the number of days between the timepoints. To measure leaf expansion, the youngest leaf of each plant was marked at the beginning of salt treatment and the length of this leaf was measured 4 times, 1, 3, 5 and 7 days after starting the stress treatment. Leaf expansion rate was expressed as the average leaf length increase per day and calculated as the difference of the leaf lengths at day 7 and day 1 divided by the number of days between these measurements (expressed as cm/day). Leaf senescence was measured by visual scoring of all leaves of each plant 17 days after starting the salt treatment. Leaf senescence scale is from 1 to 9 according the percentage of senescence area (1 =no senescence, 3 =senesced area 1-30%, 5 =senesced area 30-60%, 7 =senesced areas 60-90%, 9 =senesced area > 90%). At harvest, 17 days afer starting the stress treatment, all seedlings from the control and salt treatments were separated into shoots and roots. Plant shoot fresh weight was measured immediately at harvest. Both plant parts were dried separately in a forced-air oven at 70˚C for 2 days, and the dry weight was measured.

2.5 Ion chromatography

For determination of the ion concentrations in the shoots and roots of each genotype, four replicated samples per genotype were ground to fine powder using a hammer mill with 1 mm sieve following the protocol described by Nguyen et al. (2013). Dry leaf and root powders (25±1 mg) were ashed at 575˚C for 5 hours. Ashed samples were dissolved by shaking for 30 minutes in 1 ml 3M formic acid at 99˚C and then diluted with 9 ml MiliQ water. The samples were shaken again at 80˚C for another 30 minutes. A final 500x dilution was subsequently prepared by mixing 0.2 ml sample solution with 9.8ml MiliQ to assess the Na^+, K^+, Cl^- and Ca^{2+} content of each root and leaf sample using Ion Chromatography (IC) system 850 Professional, Metrohm (Switzerland).

2.6 Statistical analysis of phenotypic data

Analysis of variance (ANOVA) was done in a split plot design using Genstat 15th version. The four hydroponics units contained four replicated whole plots (schematically represented in Supplementary Figure 1). The whole plots were divided in two split plots of two adjacent units. The two treatments were assigned to one of the two units in a split plot. Each split plot contained 6 adjacent containers as a block (2 blocks per unit, and 4 blocks per treatment). Within each block, genotypes were randomly distributed. The growth rate and leaf expansion of each genotype in control and saline conditions were compared by student's T-test. Correlation coefficients (r) among all the parameters were calculated. All statistical analyses were performed using the statistical software package Genstat 15th edition (VSN International Hemel Hempstead, UK).

3 Results

3.1 Growth responses to salinity stress

In a pilot experiment, two genotypes (OPM-13 and OPM-38) were grown on hydroponics at 3 different salt conditions (0, 125, and 250 mM NaCl). Growth of these *Miscanthus* genotypes was already affected at 125 mM (shoot dry weight was reduced by 24% and 68% for OPM-38 and OPM-13, respectively, and 36% and 63% at 250 mM NaCl). At both salinity levels, Na^+ and Cl^- concentrations of the shoots were significantly increased (Supplementary Table 2). The high salt concentration of 250 mM seriously damaged the seedlings, which may confound the physiological interpretation of ion concentration data in relation to ion homeostasis. We concluded that a salt stress of 150 mM NaCl of the plants would affect growth of the plants considerably but inflict only limited damage. Therefore, we chose a salt stress level of 150 mM NaCl for identifying salt tolerant genotypes and traits contributing to salt tolerance.

The 70 genotypes showed a wide variation in response to 150 mM NaCl salt treatment. There were significant differences in leaf expansion, growth rate, shoot fresh weight, shoot dry weight, root dry weight, root length, the number of leaves and senescence score between the 70 genotypes ($P < 0.001$) and between control and salt treatment ($P < 0.001$) (Figure 1a - 1g). The reduction under saline conditions compared to control conditions for expansion of young leaves and growth rate in plant height was 27% and 54%, respectively. The average shoot dry weight decreased by 58% from 1.83 g under control conditions to 0.77 g under salt stress conditions. The average root dry weight was also decreased but to a lesser extent, from 0.57 g in control conditions to 0.45 g under salt stress. The average number of leaves was reduced from 3.8 to 2.6 as a result of salt stress, and senescence was increased around 1.5 fold at harvest in salt-stressed plants.

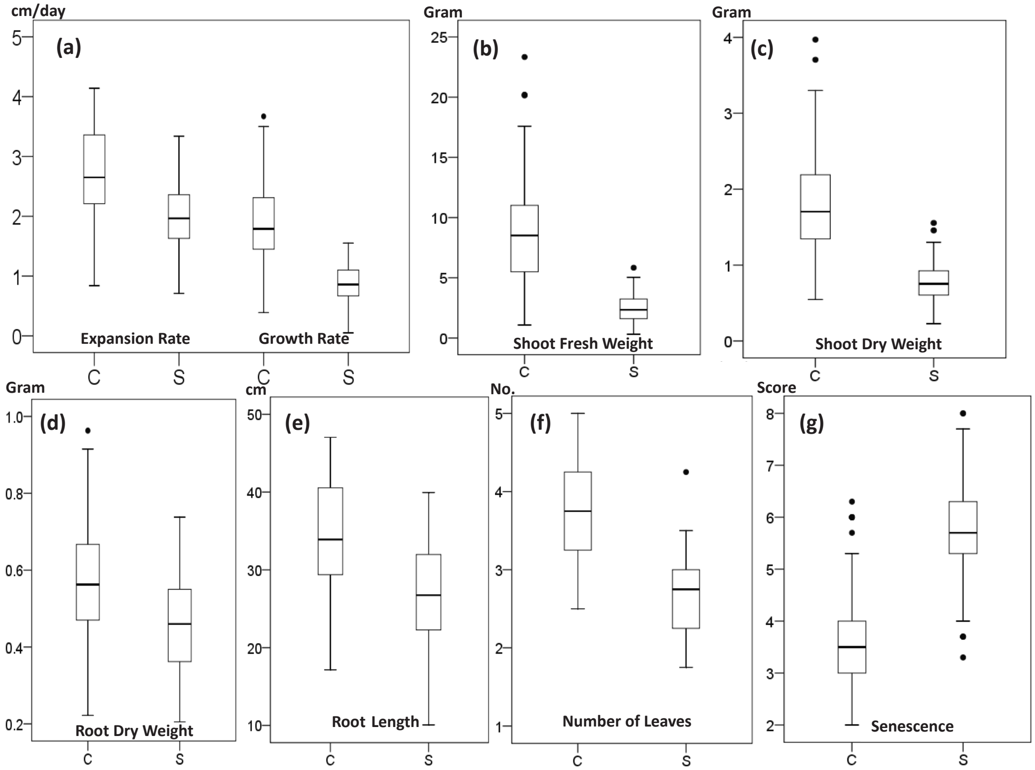

Figure 1. Box plots of growth trait data of *Miscanthus* under 0 mM NaCl (C) and 150 mM NaCl (S): Expansion and growth rate (a), shoot fresh weight (b), shoot dry weight (c), root dry weight (d), root length (e), number of leaves (f), and senescence (g). Box edges show upper and lower quartile and the median is shown in the middle of the box. Mild outliers are shown as dots.

3.2 Growth rates

The height of the salt treated plants was reduced 14-88% while the growth rate was decreased from 41-86% in the 70 genotypes. The growth rate of the seedlings was highly correlated to height both under salt (r =0.81) and control conditions (r =0.94). This trait also showed significant correlation with shoot dry weight under salinity (r =0.68) and control conditions (r =0.76). The growth rate of 22 genotypes was not significantly different at early stages between control and salt conditions (Table 2).

3.3 Leaf expansion rates

The leaf expansion rate of the 70 genotypes was on average reduced by 27% from 2.67 cm/day under control conditions to 1.96 cm/day under saline conditions. Expansion rate of the second young leaf showed a more marked difference between the salt-treated and control seedlings than the flag leaf. There were significant effects for genotype ($p < 0.001$), treatment ($p < 0.001$) and genotype by treatment interaction ($p = 0.004$) for leaf expansion rate (Table 2). Expansion rate differences between control and salt-treated genotypes ranged from 3% to 48%. In 46 genotypes, the leaf expansion rate was not significantly different from control. Leaf expansion rate significantly correlated with shoot dry weight under salinity ($r = 0.86$) and control conditions ($r = 0.82$).

Table 2. Plant growth rate (plant height increase) and leaf expansion rate of leaves of *Miscanthus* genotypes grown on hydroponics at 0 mM NaCl and 150 mM NaCl.

	Growth Rate (cm/day)					Expansion Rate (cm/day)				
Genotype	0 mM		150 mM		Sig.	0 mM		150 mM		Sig.
	Mean	S.D.	Mean	S.D.		Mean	S.D.	Mean	S.D.	
OPM-4	2.31	1.08	0.56	0.38	*	3.39	0.62	2.38	0.71	
OPM-5	2.98	0.36	1.38	0.31	***	3.66	0.69	2.92	0.76	
OPM-6	2.56	0.43	0.68	0.26	***	3.91	1.34	2.06	0.15	*
OPM-7	2.00	0.57	1.43	0.22		2.05	0.76	2.47	0.23	
OPM-8	2.29	0.45	0.53	0.25	***	2.46	0.95	1.54	0.24	
OPM-9	1.77	0.80	0.57	0.39	*	1.87	0.80	1.64	0.57	
OPM-10	1.51	1.13	0.71	0.50		3.28	1.29	2.36	0.95	
OPM-11	2.52	0.36	1.55	0.34	**	3.95	0.54	3.13	0.52	
OPM-13	1.17	0.64	0.61	0.44		2.18	0.32	1.62	0.39	
OPM-16	1.99	0.89	0.90	0.43		3.62	0.93	2.23	0.40	*
OPM-19	3.50	0.78	1.36	0.25	**	4.14	0.19	2.59	0.24	***
OPM-20	2.67	0.72	1.06	0.95	*	3.89	0.61	2.67	0.61	*
OPM-24	3.10	1.27	0.78	0.47	*	3.36	1.01	2.00	1.07	
OPM-26	1.40	0.80	0.38	0.39		1.52	0.35	1.93	0.64	
OPM-30	1.81	0.52	0.86	0.47	*	2.65	0.58	1.76	0.15	*
OPM-31	1.14	0.32	0.60	0.18	*	1.94	0.71	1.67	0.44	
OPM-32	3.67	0.41	1.28	0.48	***	3.68	0.97	2.78	1.00	
OPM-33	1.11	0.31	0.42	0.12	**	1.53	0.69	0.99	0.27	
OPM-34	1.28	0.55	0.75	0.12		1.34	0.39	1.20	0.30	

(Continued)

Table 2. Continued

Genotype	Growth Rate (cm/day)				Sig.	Expansion Rate (cm/day)				Sig.
	0 mM		150 mM			0 mM		150 mM		
	Mean	S.D.	Mean	S.D.		Mean	S.D.	Mean	S.D.	
OPM-37	2.27	1.00	0.95	0.52		2.82	1.28	2.37	0.70	
OPM-41	1.47	0.28	0.71	0.33	*	2.44	0.11	1.51	0.44	**
OPM-42	1.49	0.50	0.91	0.17		2.35	0.65	2.01	0.33	
OPM-44	0.81	0.68	0.40	0.30		1.81	0.49	1.65	0.31	
OPM-45	1.47	0.57	0.38	0.34	*	1.30	0.71	0.95	0.28	
OPM-47	0.39	0.16	0.05	0.03	**	0.84	0.28	0.71	0.08	
OPM-48	1.78	0.12	1.08	0.26	**	2.35	0.23	2.40	0.34	
OPM-49	1.87	0.52	0.82	0.19	**	3.14	0.44	2.06	0.15	**
OPM-50	1.44	0.39	0.58	0.49	*	3.68	1.01	2.49	0.18	
OPM-56	2.24	0.30	0.89	0.84	*	3.45	0.67	2.46	0.74	
OPM-57	3.02	0.91	0.88	0.18	**	4.10	0.51	2.39	0.27	***
OPM-58	1.57	0.52	0.61	0.46	*	2.66	0.50	1.41	0.54	*
OPM-59	2.50	0.05	1.17	0.14	***	3.56	0.25	2.41	0.20	***
OPM-62	1.54	0.42	0.46	0.34	**	2.75	0.59	1.49	0.37	*
OPM-64	1.61	0.30	1.07	0.34		2.66	0.24	1.97	0.41	*
OPM-65	2.35	0.36	1.22	0.27	**	3.13	0.37	2.10	0.18	**
OPM-66	0.96	0.12	0.82	0.14		1.54	0.08	1.49	0.27	
OPM-67	1.23	0.63	0.47	0.38		2.67	0.86	1.89	0.19	
OPM-68	0.84	0.62	0.59	0.13		1.49	0.44	1.31	0.25	
OPM-69	2.76	0.68	1.28	0.50	*	2.31	0.94	2.12	0.50	
OPM-71	2.00	0.84	1.31	0.20		3.00	0.72	2.46	0.15	
OPM-72	1.45	0.62	1.01	0.35		2.42	0.67	1.84	0.09	
OPM-73	1.61	0.92	0.90	0.41		2.22	1.38	2.54	0.65	
OPM-74	2.24	0.26	0.86	0.46	**	2.58	0.70	1.81	0.69	
OPM-75	1.65	0.17	1.11	0.24	**	2.32	0.36	1.93	0.25	
OPM-76	1.24	0.87	0.72	0.26		2.23	0.41	2.08	0.36	
OPM-77	1.83	0.45	1.00	0.49	*	2.21	0.39	1.50	0.32	*
OPM-78	2.25	0.33	1.27	0.33	**	2.70	0.93	2.13	0.81	
OPM-79	3.39	0.51	1.21	0.84	**	4.00	1.01	3.34	0.83	
OPM-81	1.42	0.39	0.68	0.20	*	2.22	0.71	1.43	0.35	

(Continued)

Table 2. Continued

Genotype	Growth Rate (cm/day)				Sig.	Expansion Rate (cm/day)				Sig.
	0 mM		150 mM			0 mM		150 mM		
	Mean	S.D.	Mean	S.D.		Mean	S.D.	Mean	S.D.	
OPM-82	1.58	0.53	1.10	0.11		2.65	0.22	2.09	0.26	*
OPM-83	1.35	0.48	0.88	0.16		1.89	0.56	1.63	0.16	
OPM-84	1.96	0.36	1.07	0.20	**	2.85	0.41	2.31	0.12	*
OPM-86	1.80	0.20	0.83	0.28	***	2.39	0.61	1.81	0.39	
OPM-87	2.50	0.20	0.83	0.44	***	3.26	0.27	1.96	0.50	**
OPM-88	2.10	0.86	0.89	0.34	*	2.63	1.21	1.91	0.53	
OPM-89	2.48	0.14	1.15	0.08	***	3.65	0.24	2.07	0.39	***
OPM-90	1.80	0.22	0.78	0.05	***	2.88	0.38	1.96	0.10	**
OPM-91	1.78	0.45	0.74	0.38	*	2.82	0.57	1.69	0.48	*
OPM-92	2.70	0.29	1.46	0.34	***	3.74	0.38	2.35	0.16	***
OPM-94	0.88	0.42	0.50	0.38		0.94	0.58	0.87	0.30	
OPM-96	2.08	0.32	0.76	0.41	**	3.29	0.27	1.72	0.52	**
OPM-97	1.96	0.40	1.22	0.16	*	3.34	0.24	2.17	0.30	***
OPM-98	1.61	0.48	1.06	0.47		2.24	0.82	1.84	0.25	
OPM-99	1.58	0.21	0.89	0.10	***	2.65	0.39	2.25	0.75	
OPM-100	1.76	0.40	0.78	0.49	*	1.61	0.56	1.37	0.41	
OPM-103	1.11	0.35	0.53	0.46		1.56	0.43	1.20	0.24	
OPM-104	1.45	0.38	0.67	0.30	*	2.21	0.46	1.70	0.49	
OPM-107	3.00	0.27	1.14	0.36	***	3.78	0.36	2.24	0.32	***
OPM-108	1.42	0.28	0.85	0.25	*	1.80	0.32	1.39	0.14	
OPM-109	2.90	0.59	1.54	0.47	*	3.51	0.95	2.41	0.59	

*; **; ***: significant at $P<0.05$; 0.01; 0.001 respectively

3.4 Na^+ accumulation in leaves

The 70 genotypes showed large differences in leaf Na^+ concentration of salt-stressed plants, from 4.25 mg/g in OPM-59 to 47.22 mg/g in OPM-47, and the K^+/Na^+ ratio ranged from 5.39 in OPM-59 to 0.49 in OPM-47 (Figure 2 and Supplenmentary Figure 2). Of the 6 genotypes with the highest Na^+ concentrations in the leaves (OPM-47, 49, 57, 66, 67, and 94), OPM-49 and 57 had a relatively high tiller number and low percentage of dead leaves and OPM-57 had slightly higher than average biomass (Table 3.). This indicates that these genotypes may utilize a tissue tolerance mechanism, possibly by accumulation of Na^+ in vacuoles. On the other hand, some genotypes showed low shoot sodium concentrations under salt stress. Six genotypes (OPM-4, 32, 37, 59, 69, and 71) not only showed the lowest Na^+ concentration but also had the highest K^+/Na^+ ratio in leaves. Additionally, these genotypes demonstrated less senescence on leaves compared with the high-Na^+ genotypes, relatively high biomass, and low leaf Na^+ /root Na^+ ratio. This indicates that these genotypes may utilize a shoot exclusion mechanism under saline conditions. Among these, OPM-37 was relatively tolerant and it also had the highest biomass of all genotypes under saline conditions (Table 4.).

Table 3. Trait comparisons of 6 *Miscanthus* genotypes with high leaf Na^+ ion concentrations under salt stress, grown at 150 mM NaCl on hydroponics.

Genotype	OPM-47	OPM-49	OPM-57	OPM-66	OPM-67	OPM-94	Average of 70 genotypes
Tiller number	0	2.25	2	1.25	2.25	0	1.73
Dead leaves (%)	50	32	35	40	40	56	28
Leaf Na^+ (mg/g)	47.22	30.36	26.32	33.62	24.35	20.66	12.55
Root Na^+ (mg/g)	56.5	32.85	33.33	50.99	34.58	29.81	37.23
Leaf K^+ (mg/g)	20.45	21.44	21.66	19.81	13.69	17.58	20.67
Biomass (g)	0.23	0.63	0.82	0.35	0.64	0.31	0.77
Salt tolerance (%)	41	44	29	41	54	50	43
K^+/Na^+ in leaf	0.43	0.71	0.82	0.59	0.56	0.85	2.08
Leaf Na^+/Root Na^+	0.84	0.92	0.79	0.66	0.70	0.69	0.34

Table 4. Trait comparisons of 6 *Miscanthus* genotypes with low leaf Na^+ concentrations under salt stress, grown at 150 mM NaCl on hydroponics.

Genotype	OPM-4	OPM-32	OPM-37	OPM-59	OPM-69	OPM-71	Average of 70 genotypes
Tiller number	2.8	1	3	1.3	1.8	0	1.7
Dead leaves (%)	29	20	23	22	20	24	28
Leaf Na^+ (mg/g)	7.76	5.19	6.27	4.25	6.27	4.52	12.55
Root Na^+ (mg/g)	40.4	37.64	30.04	39	43	42.45	37.23
Leaf K^+(mg/g)	34.05	28.53	25.68	22.17	25.85	22.41	20.67
Biomass (g)	0.79	1.13	1.56	0.96	0.88	1.05	0.77
Salt tolerance (%)	33	34	49	46	47	46	43
K^+/Na^+ in leaf	4.39	5.50	4.01	5.22	4.12	4.96	2.08
Leaf Na^+/Root Na^+	0.19	0.14	0.21	0.11	0.15	0.11	0.34

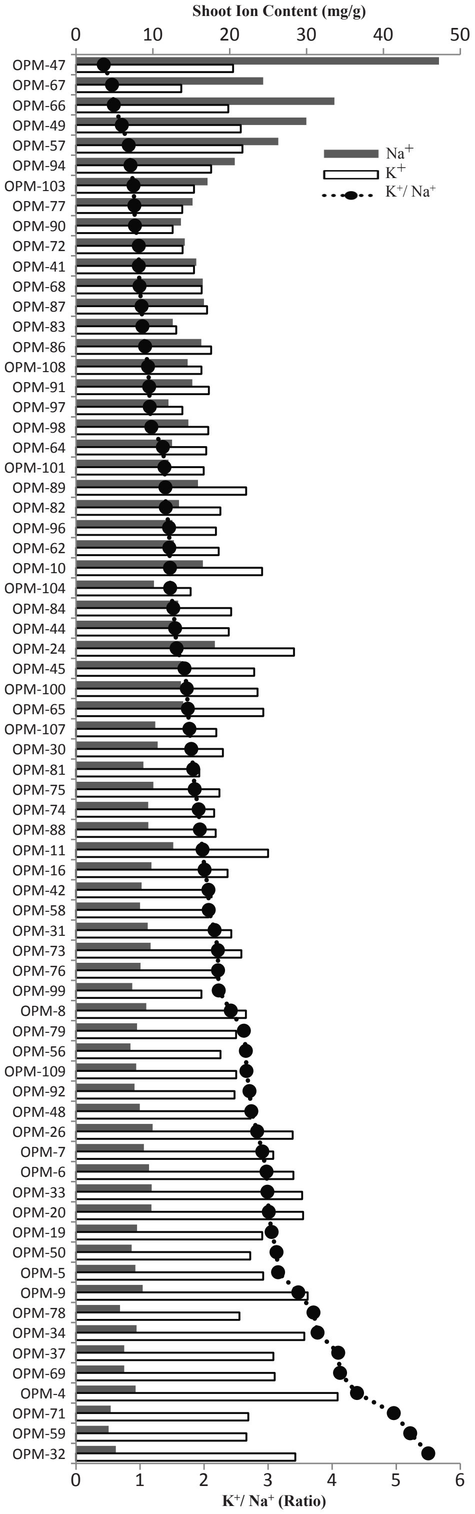

Figure 2. Shoot Na^+ and K^+ concentration (gray and white bars, respectively) and shoot K^+/Na^+ ratio (line-scatter plot) in leaves of 70 *Miscanthus* genotypes grown under saline conditions (150 mM NaCl).

3.5 Ion homeostasis change to salinity stress

The boxplots in Figures 3a and 3b show the genotypic variation of the ion contents in both shoots and roots under control and salt conditions. There were significant differences in the ion concentrations ($P < 0.001$) in shoots and roots of 70 genotypes under control and salt treatment ($P < 0.001$). The interaction between genotypes and treatments was significant ($P < 0.001$) for both Na^+ and Cl^- concentration under salt stress. In both shoots and roots, the Na^+ and Cl^- concentrations increased significantly under salt stress ($P < 0.001$), while $[K^+]$ and $[Ca^{2+}]$ decreased at 150 mM NaCl. In the leaves, Na^+ and Cl^- concentrations increased 4.6-fold and 3.1-fold under salt treatment, accumulating to 12.55 mg/g for Na^+ and 18.07 mg/g for Cl^- (Figure. 3a) but K^+ and Ca^{2+} concentrations in the shoots under saline conditions were 0.5- and 0.6-fold lower than those under control conditions. In the roots, Na^+ and Cl^- concentrations showed 13- and 5-fold increases under salt treatment, respectively accumulating to 37.23 mg/g for Na^+ and 19.66 mg/g for Cl^- (Figure. 3b) while both K^+ and Ca^{2+} concentrations decreased by 50% compared with those under control conditions. Under salt stress, Na^+ concentration in the roots was much higher than in shoots (3.6 fold), while Cl^- concentration in roots was slightly higher than in shoots (1.23 fold). This indicates that these genotypes may have an active mechanism to keep the Na^+ concentration low in the shoots.

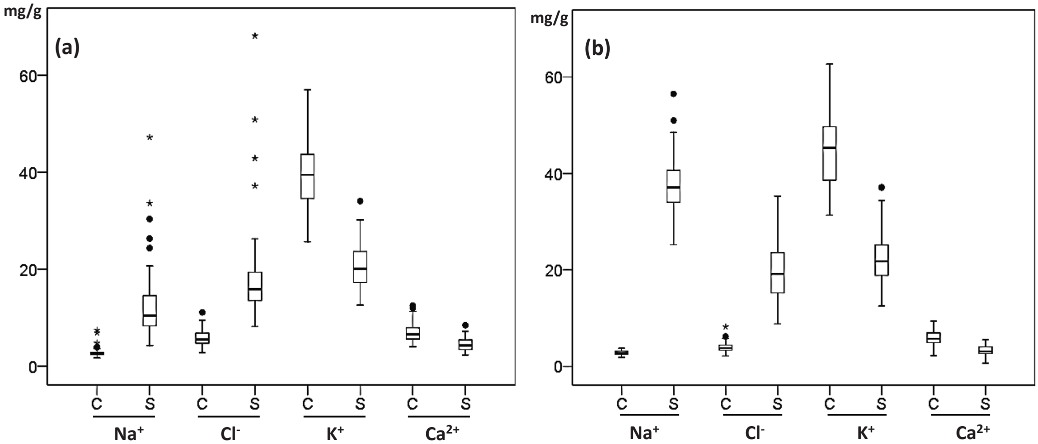

Figure 3. Box plots of ion concentrations of leaves (a) and roots (b) of 70 *Miscanthus* genotypes in control (C) and salt (S) conditions. Box edges show upper and lower quartile and the median is shown in the middle of the box. Mild outliers are shown as dots and extreme outliers shown as stars.

3.6 Salt tolerant genotypes

Salt tolerance was assessed as the percentage of shoot dry weight under saline relative to control conditions. The set of seventy genotypes grown at 150 mM NaCl in the hydroponic system showed large variation for salt tolerance, from 26% for OPM-24 to 69% for OPM-31 (Figure 4. and

Supplenmentary Figure 3). Salt tolerance of the commercial genotype OPM-9 (*Miscanthus × giganteus*) was 42%. The shoot dry weight in salt stress varied from 0.23 g to 1.56 g, and from 0.55 g to 3.97 g under control conditions. The reduction in shoot dry weight ranged from 30% to 73%. It is interesting to note that the genotypes with high salt tolerance (over 50%, less than 50% reduction in biomass) generally had relatively low biomass under control conditions. The top 10 genotypes for salt tolerance had less biomass (1.48 g) compared to overall average (1.83 g) under control conditions but the biomass was slightly higher than average (0.84 g vs 0.77 g) under salt stress (Supplementary Table 3). Those genotypes therefore were the most tolerant, but typically not the most productive under control conditions. The top 10 genotypes with high yield had on average more biomass under control conditions (2.99 g vs 1.83 g) and more biomass compared to the overall average under salt stress (1.23 g vs 0.77 g). These genotypes were still more productive under saline conditions, even if they were less tolerant to salinity (Supplementary Table 3). The genotype OPM-37 seemed to be interesting because it has the highest yield (1.56 g) under salt stress, is among the higher producers (3.16 g) under control conditions and is relatively salt tolerant (49%).

3.7 Associations between growth traits and salt

Correlations between the different physiological traits and ion concentrations are given in Table 5. A highly significant negative correlation of Cl^- and Na^+ concentrations in shoots was found with growth traits (shoot dry weight, shoot fresh weight, root dry weight, and root length) under salt stress. Leaf Cl^- and Na^+ concentrations were negatively correlated ($P<0.001$) to the shoot biomass ($r = -0.43$ and -0.53, respectively) at 150 mM NaCl. Under salt treatment, there was a high correlation between Cl^- and Na^+ concentrations in both leaves ($r = 0.94$) and roots ($r = 0.66$) but under control conditions there was only a weak correlation in leaves ($r = 0.26$) and no significant correlation in roots. The shoot dry weight was positively correlated with leaf expansion rate, root dry weight, growth rate, and root length under salt stress ($r = 0.86$, 0.85, 0.68 and 0.62, respectively). The correlation between K^+ and Na^+ concentrations in leaves and roots were not significant under salt stress while there was weak correlation for these traits in both leaves ($r = 0.48$) and roots ($r = 0.44$) under control conditions. However, the K^+ concentration in leaves was positively correlated with shoot fresh weight ($r = 0.41$) and weakly correlated with shoot dry weight ($r = 0.30$) at 150 mM NaCl, similar to the correlations at 0 mM NaCl ($r = 0.4$ and 0.28, respectively). The ratio of K^+/Na^+ was positively ($P < 0.001$) related to the shoot biomass ($r = 0.56$) in all genotypes under salt treatments but it was weak ($r = 0.31$) under control conditions.

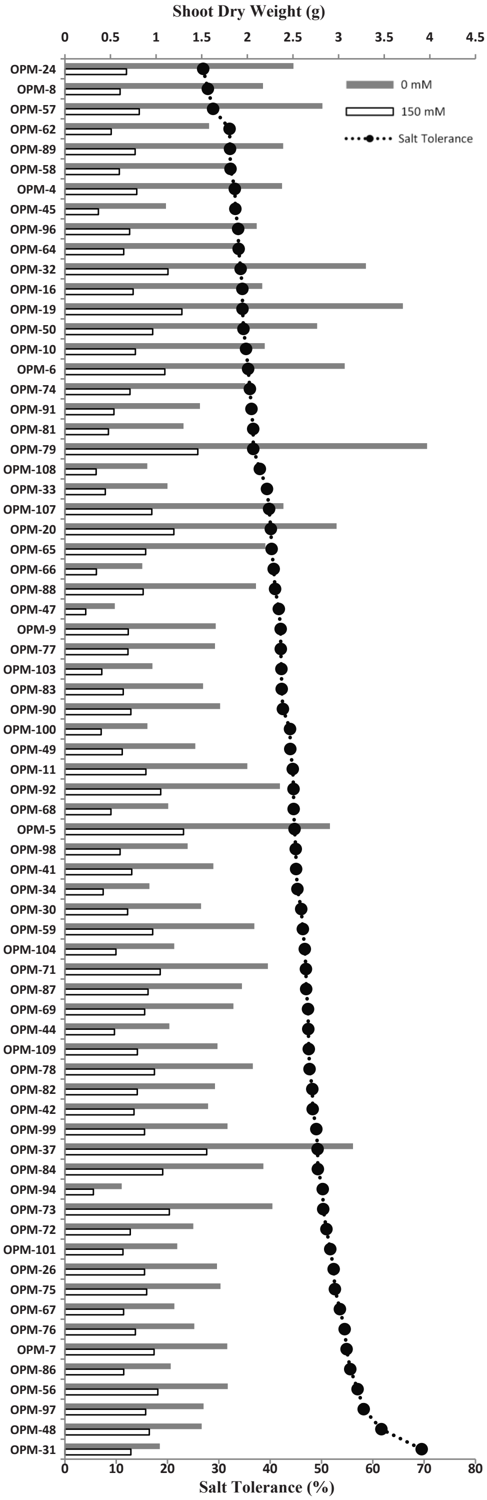

Figure 4. Shoot dry weight (bars) and salt tolerance (line-scatter plot, calculated as the ratio of shoot dry weight under salt stress and shoot dry weight under control conditions) of 70 genotypes of *Miscanthus* grown in a hydroponics system at 0 mM NaCl (gray bars) and 150 mM NaCl (white bars).

Table 5. Pearson correlations between the traits under salt stress (left lower triangle) and control (right upper triangle). The left upper to right lower corner diagonal indicates the correlation between trait values for control and saline conditions.

	Cl^- leaf	Na^+ leaf	K^+ leaf	Cl^- root	Na^+ root	K^+ root	K^+/Na^+ leaf	K^+/Na^+ root	Exp	GR	Height	SFW	SDW	RDW	RL	Tiller No.	Leaf No.	Sen	DL
Cl^- leaf	0.09	0.26	0.76	-0.09	0.31	0.00	0.26	-0.24	0.20	0.52	0.44	0.45	0.35	0.09	0.28	0.13	0.63	0.01	0.21
Na^+ leaf	0.94	0.01	0.48	-0.21	0.20	0.17	-0.47	-0.08	-0.07	0.03	0.00	-0.02	-0.09	-0.09	0.04	-0.12	0.22	-0.04	0.05
K^+ leaf	0.00	-0.23	0.69	-0.26	0.31	0.20	0.18	-0.12	0.18	0.44	0.34	0.40	0.28	0.07	0.20	0.19	0.56	0.08	0.23
Cl^- root	0.25	0.11	0.31	0.14	0.11	0.05	-0.15	-0.09	-0.13	-0.24	-0.26	-0.22	-0.19	-0.04	-0.10	-0.22	-0.25	0.18	0.21
Na^+ root	0.37	0.26	0.13	0.66	0.23	0.44	-0.04	-0.55	0.09	0.26	0.20	0.17	0.11	0.01	0.04	0.02	0.12	0.00	0.10
K^+ root	-0.13	-0.16	0.28	0.37	0.07	0.29	-0.05	0.44	-0.17	-0.04	-0.11	-0.09	-0.10	-0.04	-0.14	0.06	-0.07	0.17	0.14
K^+/Na^+ leaf	-0.43	-0.65	0.67	0.20	0.02	0.24	0.14	0.13	0.21	0.39	0.33	0.35	0.31	0.12	0.06	0.28	0.23	-0.03	-0.04
K^+/Na^+ root	-0.24	-0.20	0.15	-0.03	-0.48	0.82	0.16	-0.01	-0.19	-0.21	-0.21	-0.16	-0.12	-0.05	-0.17	0.11	-0.06	0.11	0.02
Exp	-0.35	-0.43	0.28	-0.21	-0.20	0.01	0.48	0.06	0.80	0.74	0.85	0.81	0.82	0.81	0.60	0.49	0.35	0.03	-0.05
GR	-0.48	-0.51	0.16	-0.09	-0.19	0.19	0.44	0.22	0.74	0.73	0.94	0.83	0.76	0.53	0.47	0.34	0.61	-0.04	-0.01
Height	-0.45	-0.52	0.25	-0.21	-0.22	0.01	0.49	0.06	0.95	0.81	0.87	0.90	0.86	0.66	0.55	0.42	0.61	-0.03	-0.04
SFW	-0.37	-0.48	0.41	-0.08	-0.12	0.14	0.57	0.13	0.84	0.73	0.88	0.88	0.98	0.74	0.56	0.52	0.66	-0.02	-0.03
SDW	-0.43	-0.53	0.30	-0.17	-0.18	0.01	0.56	0.05	0.86	0.68	0.90	0.92	0.85	0.80	0.59	0.53	0.61	-0.02	-0.06
RDW	-0.42	-0.47	0.09	-0.20	-0.19	0.04	0.40	0.08	0.81	0.65	0.83	0.80	0.85	0.81	0.73	0.38	0.27	0.00	-0.11
RL	-0.34	-0.34	-0.03	-0.18	-0.18	-0.16	0.23	-0.11	0.61	0.60	0.70	0.59	0.62	0.72	0.72	0.21	0.36	-0.01	-0.03
Tiller No.	-0.18	-0.20	0.16	-0.44	-0.35	-0.29	0.11	-0.09	0.46	0.19	0.44	0.39	0.44	0.37	0.40	0.45	0.31	0.12	0.07
Leaf No.	-0.18	-0.32	0.48	0.07	0.13	0.10	0.42	0.00	0.29	0.29	0.32	0.47	0.35	0.22	0.28	0.25	0.68	-0.01	0.09
Sen	0.41	0.54	-0.33	-0.16	0.15	-0.27	-0.59	-0.27	-0.25	-0.33	-0.30	-0.33	-0.29	-0.27	-0.22	-0.06	-0.13	0.12	0.89
DL	0.55	0.69	-0.32	0.08	0.15	-0.12	-0.61	-0.11	-0.49	-0.49	-0.55	-0.57	-0.56	-0.52	-0.41	-0.32	-0.47	0.78	0.19

Senescence (Sen), Dead leaves (DL), Expansion rate (Exp), Growth rate (GR), Shoot Fresh Weight (SFW), shoot dry weight (SDW), Root dry weight (RDW), Root length (RL).

4 Discussion

Bioenergy crops are an important alternative to fossil fuel, and a valuable addition to other alternative forms of energy (Brosse et al., 2012). Growing these crops on underutilized, marginal soils like saline soils would avoid competition with food crops for agricultural lands. The potential for improvement of *Miscanthus* for salinity tolerance still remains to be established, as most research has focused only on *Miscanthus* × *giganteus* (Plazek et al., 2014; Stavridou et al., 2016) and genetic diversity for salinity tolerance of *Miscanthus* germplasm is largely unknown. The current study evaluated seventy *Miscanthus* genotypes under salt stress and showed that broad diversity for salt tolerance and salt tolerance traits is present in *Miscanthus*. Several highly salt tolerant genotypes utilizing different mechanisms can be considered as valuable breeding materials.

4.1 Screening system

A reliable screening system for salt tolerance traits is essential, as uniform exposure of plants to salt stress is hard to establish and control in field experiments (Munns and James, 2003; Almeida et al., 2016). Hydroponic systems supply uniform conditions for the root environment, and have a high capacity of genotypes at the same time (Nguyen et al., 2013; Chan-Navarrete et al., 2014). Using such a system, traits and QTLs contributing to variation in salt tolerance in barley were already successfully identified (Long et al., 2013; Nguyen et al., 2013), and to variation in nitrogen use efficiency in spinach (Chan-Navarrete et al., 2016). It is important to keep in mind however that factors like soil texture and composition that in the field also may influence salinity tolerance do not play a role in this type of system. Also, root properties related to soil traits and exploration of the soil will have a different impact on growth and yield. Another limitation of hydroponics evaluation is that it only allows screening of relatively young plants. Nevertheless, given the difficulty to maintain uniform screening conditions in a large population in the field (Tavakkoli et al., 2012), hydroponics provides a highly useful alternative. It is a fast and uniform way to identify high potential genotypes with interesting salt tolerance traits that particularly relate to ion homeostasis and other cellular tolerance mechanisms, like osmotic adjustment and scavenging of reactive oxygen species (ROS). Indeed, several studies on salt tolerance using hydroponics systems found correlations between salt tolerance and Na^+ and K^+ concentrations in shoot (Munns and James, 2003; Jaarsma et al., 2013; Platten et al., 2013). Similarly, we identified several salt tolerant genotypes in our hydroponics-based screening with low Na^+ concentrations in the leaves (Table 4). These are likely to utilize Na^+ exclusion mechanisms and may be useful genitors for salinity tolerance breeding programs. Genotype OPM-37 was even among the highest biomass producers both under control and salt conditions, and should be evaluated under field conditions as a potential high producing genotype on saline soils.

4.2 Mechanisms and useful traits

When grown in saline soils, plants are exposed to osmotic stress and ionic stress (Ashraf and Harris, 2004; Munns and Tester, 2008). Since osmotic and ionic stress both decrease yield and growth rate, improving salt tolerance in crops needs to take into account both osmotic tolerance and ion exclusion (Genc et al., 2010). Osmotic tolerance appears to contribute more to salt tolerance than avoiding ion toxicity in cultivated wheat and in barley (Rengasamy, 2010a). Leaf expansion is considered a good indicator for osmotic tolerance (Rajendran et al., 2009; Farouk, 2011). In our tested *Miscanthus* genotypes, leaf expansion showed highly significant correlation with shoot dry weight ($r = 0.86$) under salinity. The relatively high variation in the leaf expansion measurements may be caused by the relatively high variation in youngest leaf length between replicates of a genotype at the start of the measurements. This may be avoided by using more replicate plants and selecting only the plants with youngest leaves of comparable lengths, but that would require either a higher capacity (number of plants) of plants, or a reduction in the number of genotypes.

The most likely candidate genotypes to have osmotic tolerance may be the ones that have both limited reduction in both leaf expansion rate and in growth rate at early stages of the stress. Forty-six of the *Miscanthus* genotypes evaluated in this study showed no significant difference in expansion rate and twenty-two genotypes had no significant difference in growth rate as a result of salt stress (Table 2). Nineteen genotypes (OPM-7, 10, 26, 34, 37, 42, 44, 66, 67, 68, 71, 72, 73, 76, 83, 94, 98, 13 and 103) had both little reduction of leaf expansion and height, which would imply that more than 25% of the tested genotypes may have some level of osmotic tolerance that minimizes the early effects of salinity.

Ion toxicity is induced by prolonged salinity due to the accumulation of ions in plant tissues, especially in the leaves (Munns and James, 2003). The leaf blades are more sensitive to ion toxicity than the roots, so adapting ion homeostasis to keep a relatively low Na^+ concentration in the leaf is important (Munns and Tester, 2008). Under salt stress, high Na^+ concentrations interfere with K^+ uptake and K^+ function (Shabala and Cuin, 2008). Maintaining a high K^+ concentration at relatively high Na^+ levels is therefore another important mechanism under salt stress, and the K^+/Na^+ ratio is considered an indicator of salt tolerance (Munns and James, 2003; Krishnamurthy et al., 2007). In a large-scale screen of 69 barley cultivars, 90% of the genotypes used an active K^+ maintenance mechanism to retain cytosolic K^+ concentrations, while 10% achieved this indirectly by efficiently excluding Na^+ from shoot (Chen et al., 2007; Schmer et al., 2008). In our evaluation, the K^+/Na^+ ratio was not only positively correlated (r =0.56) to shoot dry weight in the 70 tested genotypes, but also negatively correlated (r =-0.59) to senescence under salt stress. Only twelve of the 70 genotypes had a K^+/Na^+ ratio of less than 1 (Figure 2), indicating that most *Miscanthus* genotypes were able to maintain a relatively high K^+ concentration compared to the Na^+ concentration.

Six genotypes with a high K^+/Na^+ ratio (more than twice the average value) had low Na^+ concentrations in the leaves (Table 4).A low Na^+ concentration in the shoots was successfully used as selection criteria to breed for salt tolerant cultivars in wheat, barley and rice (Lin et al., 2004; Lindsay et al., 2004; Xue et al., 2009; Genc et al., 2010; Thomson et al., 2010; Munns et al., 2012). We identified a number of genotypes with low Na^+ concentrations in the shoot and high salt tolerance, suggesting that these utilize Na^+ exclusion mechanisms similar to those used for improving salt tolerance in cereals. The genotypes with the lowest Na^+ concentrations in leaves also showed the lowest Na^+ shoot/ Na^+ root ratio (Table 4) implying that Na^+ is actively excluded from the shoots.

The gene underlying the Na^+ exclusion introduced from wild relatives in both rice and wheat was shown to be a member of the Na^+-selective transporter HKT gene family. This HKT1;5 gene is expressed in parenchyma cells aligning the xylem in roots, and the HKT1;5 transporter was shown to filter Na^+ out of the xylem, thus preventing transport Na^+ from the roots to the shoots (Maser et al., 2002; Husain et al., 2003). Seven major and three minor alleles of OsHKT1;5 were identified in rice and the leaf Na^+ concentration was highly associated with *HKT1;5* allelic variation across diverse accessions (Platten et al., 2013). It is not unlikely that a *Miscanthus* HKT1;5 ortholog is responsible for the variation in Na^+ shoot concentrations in *Miscanthus*. It would therefore be worthwhile to study allelic variation and activity of this *Miscanthus* HKT1;5 ortholog under saline conditions in *Miscanthus* genotypes.

Because electrochemical balance is vital under stress, Cl^- and Na^+ uptake are often linked (Teakle and Tyerman, 2010). However, the Na^+ and Cl^- exclusion mechanisms are independent, with different genotypes having different mechanisms to regulate Na^+ or Cl^- transport (Teakle and Tyerman, 2010). For example, genotypes of *Glycine max* were more sensitive to Cl^- ion accumulation, but *G. soja* genotypes were more sensitive to high levels of Na^+ ions (Luo et al., 2005). In our *Miscanthus* genotypes, the average Cl^- root/shoot ratio was 1.23 but the Na^+ root/shoot ratio was 3.6 under salt treatment over 70 genotypes (Figure. 3). This indicates that an active mechanism to avoid accumulation of Na^+ in the leaves is relatively abundant in *Miscanthus*, and a similar mechanism for Cl^- accumulation in the shoots is much less prevalent. Nevertheless, there was a high correlation ($r = 0.94$) between Cl^- and Na^+ concentrations in leaves and both Cl^- and Na^+ had negative correlations with shoot dry weight stress, $r = -0.43$ and $r = -0.53$, respectively (Table 5). It is interesting that four genotypes (OPM-59, 71, 78 and 109) showed low Cl^- concentrations (8.14-10.09 mg/g) compared with the average (18.06 mg/g) in leaves as well as a relatively high Cl^- root/shoot ratio (0.41-0.49) compared with the average (0.98). Those genotypes may have Cl^- exclusion mechanisms (Supplementary Table 4).

In the present study, two genotypes (OPM-49 and 57) also showed more tillers and less senescence even with having high shoot concentrations of Na^+ and Cl^-. This may be indicative for a tissue tolerance mechanism, with Na^+ and Cl^- compartmentalized into the vacuoles to avoid toxic concentrations within the cytoplasm (Munns and James, 2003).

4.3 Rhizome

Root traits studied in a hydroponic system may not be representative for root characteristics in the soil and the effect on yield (Tavakkoli et al., 2012). For a perennial with a rhizome, like *Miscanthus*, this may be even more true. Chinese ryegrass *Leymus chinensis* can adapt to salt stress by accumulating Na^+ in the rhizome (Mann et al., 2013; Li et al., 2014). A similar result was found for *Miscanthus* × *giganteus* in a pot experiment; the Na^+ concentration in rhizomes was 3-fold higher in the rhizome than that in shoot under 150 mM NaCl, and plants with larger rhizomes were more tolerant than plants with small rhizomes, with lower decreases in shoot dry weight under salinity (Plazek et al., 2014). This indicates that rhizomes may play an important role in salt tolerance of perennial grasses, and obviously this component of salt tolerance can not be tested on a hydroponics system. However, keeping the limitations of the hydroponics system in mind, the advantages in terms of uniformity of plants and environmental conditions, as discussed before, can be exploited. We have shown here that identification of genetic variation for salt tolerance traits, and of mechanisms utilized by *Miscanthus* to counteract the effect of salinity can be done effectively on a hydroponics system. A selection of genotypes with varying salt tolerance properties could thus be made, and these can be used to study salt tolerance mechanisms in more detail in soil-grown plants in pots or in the field.

4.4 Preferred Genotypes

Although *Miscanthus* × *giganteus* with its high yield is the most popular commercial genotype, it has several disadvantages. Firstly, its tolerance to abiotic stress is not as high as *M. sinensis* (Clifton-Brown et al., 2001). With respect to chilling tolerance, the rhizomes of *Miscanthus* × *giganteus* cannot survive below approximately -3˚C but the hybrids of *M. sinensis* still live below -4.5˚C (Clifton-Brown and Lewandowski, 2000). Shoot dry weight of *Miscanthus* × *giganteus* in pots was reduced by 50% after 64 days at 120 mM NaCl (Stavridou et al., 2016), while *M. sinensis* accessions exhibited less than 40% reduction under the same levels of salt stress (Sun et al., 2014). In our experiment, the reduction of *Miscanthus* × *giganteus* (OPM-9) at 150 mM NaCl for 2 weeks was 57%, which was identical to the average salt tolerance in 70 genotypes. This offers opportunities for selecting and breeding genotypes that surpass *Miscanthus* × *giganteus* in salt tolerance and growth on marginal, saline soils. OPM-37 for instance had the highest yield under salt stress, and OPM-31 had the lowest reduction compared to yield under control conditions. The hybrids OPM-5 (*M. sinensis* × *M. sacchariflorus*), and OPM-7 (*M. sacchariflorus* × *M. sinensis*) used in our study had higher yield than *Miscanthus* × *giganteus* under control and salt stress as well as higher salt tolerance. These genotypes may be favorable choices for producing biomass on saline lands, and also may indicate the potential of new hybrids that combine favorable traits identified in this study. Lewandowski et al. (2016) indicated that OPM-5 and OPM-7 in a multi-location trial were not among the highest biomass producers under non-saline conditions. Whether these genotypes will be relatively good performers on saline soils remains to be established. Several genotypes had relatively high yields under both control and saline conditions, and may be preferred in soils with varying levels of salinity. These include OPM-5, OPM-6, OPM-19, OPM-20, OPM-32, OPM-37, and OPM-79. Among these seven genotypes, OPM-37 and OPM-5 have salt tolerance of 49% and 44%, respectively, just above the average (43 %). These two genotypes may show osmotic mechanisms with limited reduction of the expansion rate. OPM-37 and OPM-5 had a less than average reduction in tiller number due to salt stress, and above average tiller number (3 and 3.25 respectively, while average tiller number was 1.7) under salt stress. These two genotypes have relatively high potential to be cultivated on marginal lands.

It is important to take into account how *Miscanthus* quality is used for bioenergy when choosing optimal genotypes for growth under saline conditions, or genitors for breeding programs. A low ion content of harvested material is very important for combustion quality because the high mineral content can reduce the ash melting point and cause corrosion issues, especially K^+ and Cl^- (Brosse et al., 2012). Jorgensen (1997) showed that during harvest season (spring) the K^+ and Cl^- concentrations in *M. sinensis* were reduced by 85-95% in the normal field because of relocation of minerals to storage organs and leaching by rain. However, the potential impact on combustion properties for material grown on saline lands is largely unknown. Whether the ions accumulate in the senesced stem that is harvested still needs to be established. If the Na^+ and Cl^- accumulate in the shed leaves but not in the stems, genotypes with salt inclusion could also be considered as good genitors for breeding. If the ions still accumulate in the stems, the genotypes with salt exclusion would be preferred as starting materials for breeding; OPM-59 and OPM-71 would be good candidates, with lowest concentrations of Na^+ and Cl^- under salt stress in the shoots. Another quality aspect to consider is the cell wall; stress is known to cause changes in the cell wall composition (Le Gall et al., 2015). Drought stress reduced the cellulose content but increased the hemicellulosic polysaccharides so that available cell wall polysaccharides were more easily released as fermentable sugars during processing (van der Weijde et al., 2016). However, the interaction between cell wall composition and salt stress in *Miscanthus* is still unexplored.

5 Funding

The research received funding from the European Union consortium OPTIMISC (project ID 289159).

Chang-Lin Chen is supported by a scholarship from the Ministry of Education, Taiwan.

6 Acknowledgments

This work was performed as part of the OPTIMISC project, which received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement No. 289159. The authors gratefully acknowledge expert advise from Oene Dolstra on choice of materials, and the expert assistance with the hydroponics system of Geurt Versteeg and Unifarm. We thank IBERS and ILVO for supplying *Miscanthus* genotypes, and Christel Denneboom for help with *in vitro* culture.

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Supplementary material

Supplementary Table 1. The composition of 0.5X Hoagland solution.

Macro elements	Conc.[mM]	Micro elements	Conc.[µM]
NH_4	0.500	Fe	35.000
K	3.000	Mn	11.800
Ca	2.000	Zn	1.750
Mg	1.000	B	43.700
NO_3	6.000	Cu	0.125
SO_4	1.250	Mo	0.520
P	1.000		
Si	1.480		

Supplementary Table 2. Shoot Dry Weight (SDW), Shoot Na^+ content and Shoot Cl^- content of two genotypes evaluated in the pilot experiment.

Genotype	Treatment	SDW (g)	Shoot Na^+(mg/g)	Shoot Cl^-(mg/g)
OPM-38	0 mM	2.5 ± 0.2	2.9 ± 0.9	9.4 ± 0.95
	125 mM	1.9 ± 0.2	19.0 ± 3.2	21.2 ± 4.3
	250 mM	1.6 ± 0.2	30.8 ± 8.9	48.8 ±19.6
OPM-13	0 mM	1.9 ± 0.1	1.9 ± 2.5	7.9 ± 3.0
	125 mM	0.6 ± 0.1	8.9 ± 3.0	15.8 ± 3.0
	250 mM	0.7 ± 0.1	50.9 ± 7.4	92.1 ± 17.3

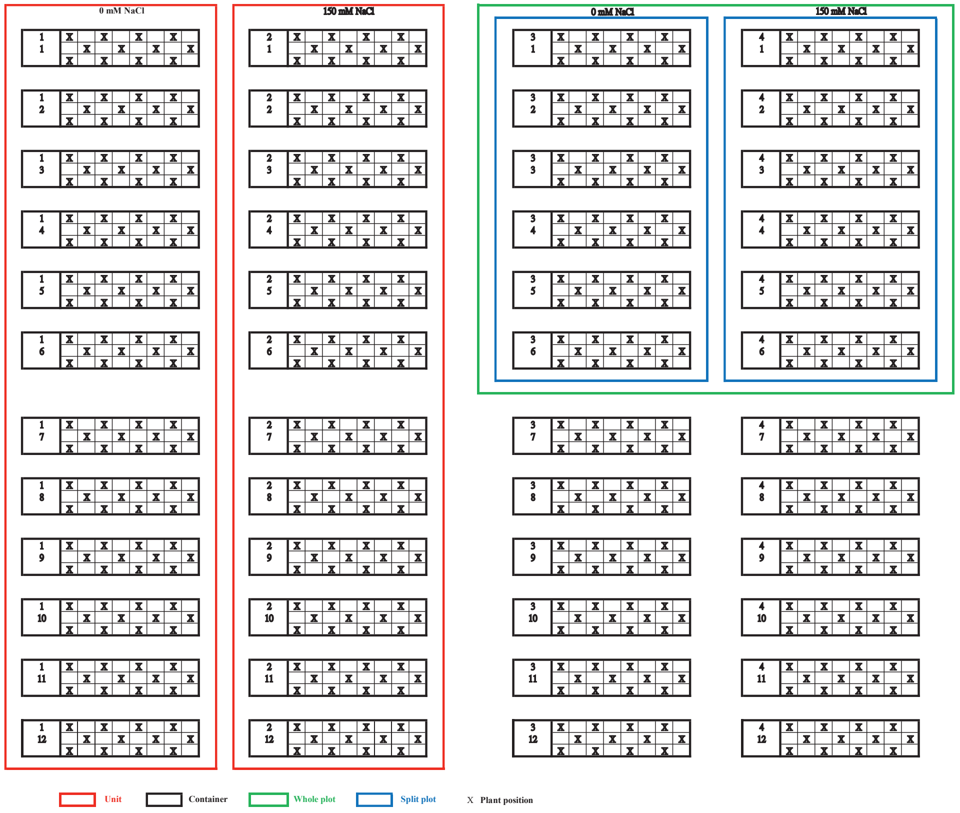
Supplementary Table 3. Shoot dry weight and Salt Tolerance of the ten genotypes with the highest salt tolerance and the highest yield under salt stress.

Genotype	Shoot dry weight (g)		Salt Tolerance (%)	Genotype	Shoot dry weight (g)		Salt Tolerance (%)
	0 mM	150 mM			0 mM	150 mM	
OPM-31	1.04	0.72	70	OPM-37	3.16	1.56	49
OPM-48	1.50	0.93	62	OPM-79	3.97	1.46	37
OPM-97	1.52	0.89	58	OPM-5	2.91	1.30	45
OPM-56	1.79	1.02	57	OPM-19	3.71	1.28	35
OPM-86	1.16	0.65	56	OPM-20	2.98	1.20	40
OPM-7	1.78	0.98	55	OPM-73	2.28	1.15	50
OPM-76	1.42	0.77	55	OPM-32	3.30	1.13	34
OPM-67	1.20	0.64	54	OPM-6	3.07	1.10	36
OPM-75	1.71	0.90	53	OPM-84	2.18	1.07	49
OPM-26	1.67	0.87	52	OPM-92	2.36	1.05	45
Average	1.48	0.84	57	Average	2.99	1.23	42

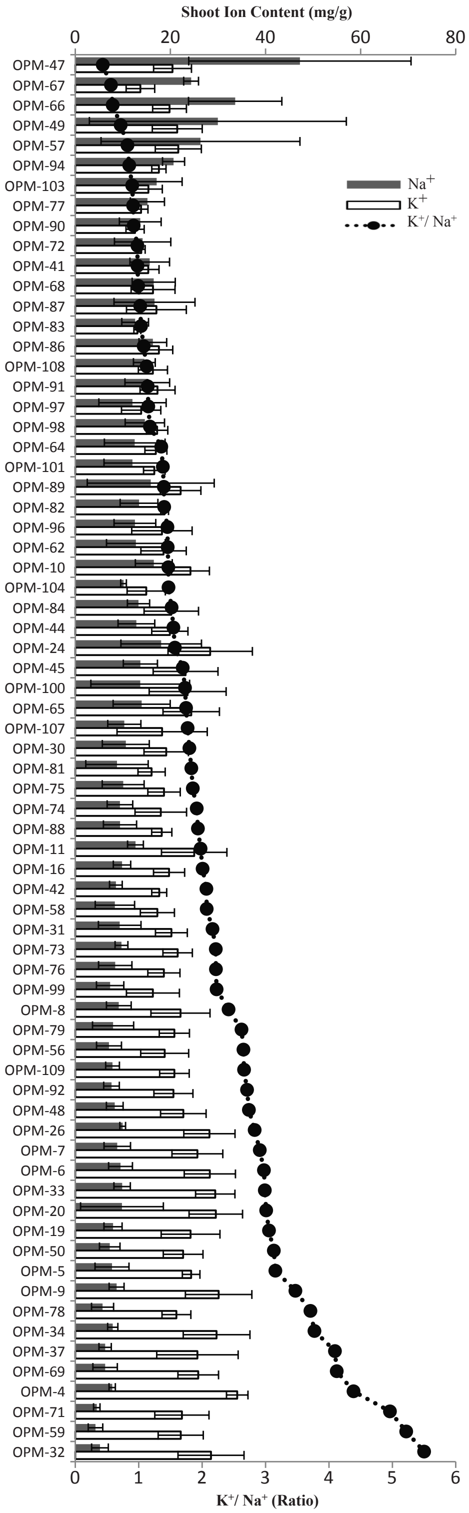
Supplementary Table 4. Trait data of four genotypes with the highest Cl$^-$ concentrations in leaves under salt stress.

Genotype	OPM-59	OPM-71	OPM-78	OPM-109	Average of 70 genotypes
Tiller number	1.25	0	1.50	1.25	1.73
Dead leaves (%)	22	24	24	24	28
Leaf Cl$^-$ (mg/g)	10.09	9.32	8.85	8.14	18.06
Root Cl$^-$ (mg/g)	23.74	19.94	18.15	19.90	19.66
Leaf K$^+$ (mg/g)	33.17	22.41	21.26	20.84	20.67
Biomass (g)	0.96	1.05	0.98	0.80	0.77
Salt tolerance (%)	46	46	47	47	43
Leaf Cl$^-$/Root Cl$^-$	0.43	0.47	0.49	0.41	0.98

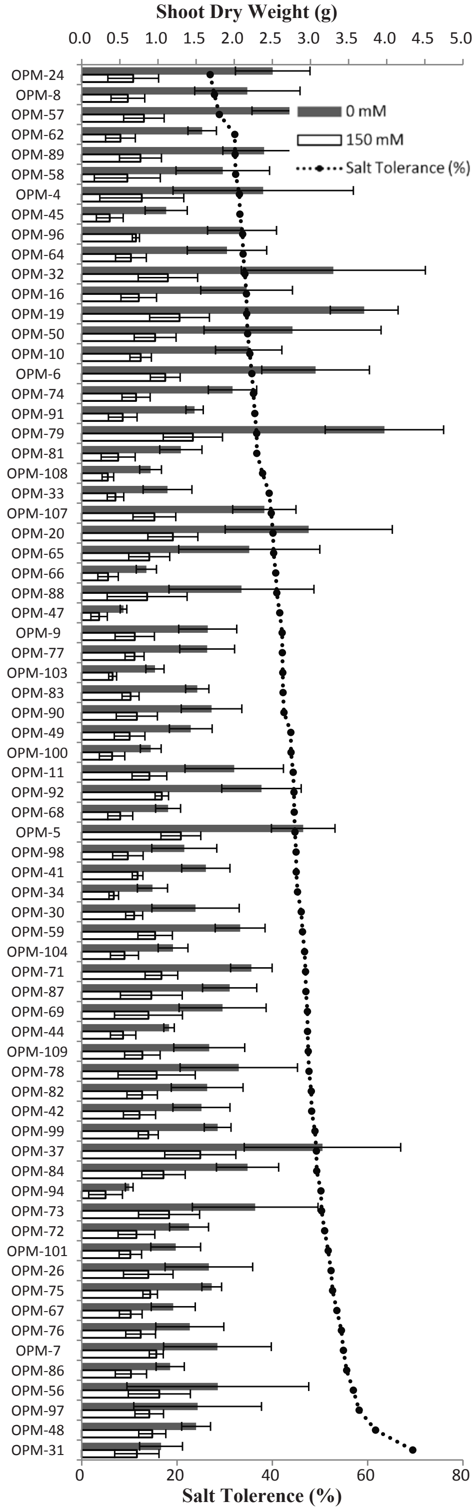
Shoot dry weight under salt stress/ shoot dry weight under control conditions) of 70 *Miscanthus* genotypes grown in a hydroponics system at 0 mM NaCl (gray bars) and 150 mM NaCl (white bars). Values are mean (n = 4).



Supplementary Figure 1. Experimental design with 4 units for control and salt treatment. Each unit contained 12 containers for 2 replications with randomly distributed *Miscanthus* genotypes. The two treatments were assigned to one of the two units in a split plot. The whole plot contained 2 split plots and each split plot contained 6 adjacent containers.



Supplementary Figure 2. Shoot Na^+ and K^+ concentration (gray and white bars, respectively) and shoot K^+/Na^+ ratio (line-scatter plot) in leaves of 70 *Miscanthus* genotypes grown under saline conditions (150 mM NaCl). Error bars indicate standard deviation (n =4).



Supplementary Figure 3. Shoot dry weight (bars) and salt tolerance (line-scatter plot, calculated as the ratio of shoot dry weight under salt stress and shoot dry weight under control conditions) of 70 genotypes of *Miscanthus* grown in a hydroponics system at 0 mM NaCl (gray bars) and 150 mM NaCl (white bars). Error bars indicate standard deviation (n = 4).

Chapter 3

Performance and salt tolerance evaluation of twelve rhizome-derived and *in vitro*-started *Miscanthus* genotypes

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Keywords: ***Miscanthus*, salt tolerance, osmotic stress, ionic stress, ion homeostasis.**

Abstract

Miscanthus is a perennial rhizomatous C4 grass with a high potential as a CO_2-neutral biofuel resource. It is relatively salt tolerant and therefore has potential to be cultivated on saline soils, avoiding competition for arable lands with other crops. By exploring the genetic variation of different *Miscanthus* genotypes under different levels of salt stress, high potential genotypes can be identified as breeding materials for improvement of salt tolerance or as genotypes that can be cultivated on saline soil. Twelve genotypes of *Miscanthus* (10 *M. sinensis* and 2 hybrids) were evaluated for salt tolerance and ion contents under three salinity levels (0, 150, and 250 mM NaCl) in pots. *In vitro* plants raised on hydroponics, and plants started from rhizomes were used in this study. The biomass of shoots and roots was slightly decreased by 150 mM NaCl treatment. At 250 mM NaCl, the biomass was more severely affected and genotypic differences were more evident, including genotypic variation for Na^+ and Cl^- accumulation in leaves. Under these higher salinity conditions, plants started from rhizomes had less senescence symptoms compared to *in vitro*-started plants even though there were no significant differences in ion contents in shoots, which might indicate that the presence of rhizomes protects the leaves against salt-induced senescence. The *M. sinensis* genotype OPM-56 had clearly lower Na^+ and Cl^- contents in the shoots than the other genotypes, demonstrating that a Na^+ exclusion mechanism was active in this genotype to prevent Na^+-accumulation in the leaves at this level of salt stress. Genotype OPM-56 also had relatively high yield under both saline and normal growth conditions as well as stable and relatively high salt tolerance, for both *in vitro* and rhizome-started plants. Therefore, genotype OPM-56 may have high potential for growth on saline soils and as a starting material for breeding programs towards salt tolerant *Miscanthus* varieties.

1 Introduction

Miscanthus, a perennial rhizomatous grass with C4 photosynthesis, originates from Southeast Asia, the Pacific islands, and tropical Africa. The genus includes 15 species and a natural interspecific hybrid, *Miscanthus* × *giganteus* (3n =3x =57) between *Miscanthus sinensis* (2n =2x =38) and *Miscanthus sacchariflorus* (2n =4x =76). *Miscanthus* × *giganteus* is the genotype that is commercially used for biomass production because of amongst others its high photosynthetic efficiency, high yield, and low input demands (Dohleman and Long, 2009; Zub and Brancourt-Hulmel, 2010; Davey et al., 2016; van der Weijde et al., 2016). However, there are several disadvantages of *Miscanthus* × *giganteus*. Firstly, this triploid hybrid does not produce viable seeds. This leads to an increase of cultivation costs because propagation from rhizomes or tissue culture is more expensive than from seeds; the cheapest way would be direct seed sowing if it will become feasible in future (Xue et al., 2015). Secondly, it is difficult to improve *Miscanthus* × *giganteus* by crossing as it is to sterile triploid. *M. sinensis* (one of the parents for *Miscanthus* × *giganteus*) however is diploid and can be crossed and can facilitate breeding. Thirdly, the yield of *Miscanthus* × *giganteus* is affected by abiotic stress. It is susceptible to frost stress compared with *M. sinensis* genotypes (Fonteyne et al., 2016a) and genotypes of *M. sinensis* with higher salt tolerance have been identified as well (Chen et al., 2017). For successful cultivation of *Miscanthus* as a bioenergy crop, it is essential to identify stress-tolerant genotypes that are better adapted to different climatic conditions and to adverse and marginal site conditions, such as cold, drought, and salinity (Lewandowski et al., 2016). *M. sinensis* has a widespread geographical distribution in terms of longitude as well as latitude with correspondingly extensive genetic diversity for environmental adaptation, and it was introduced as a potential bioenergy crop and adapted widely to Europe (Clifton-Brown et al., 2001). In Chapter 2, we evaluated genetic diversity for salt tolerance in *Miscanthus* germplasm on a hydroponics system, and identified a number of salt tolerant genotypes that most likely utilized different mechanisms for salt tolerance (Chen et al., 2017). However, the hydroponics system only allowed testing of relatively young plants for a short period. Moreover, performances of plants grown in hydroponics may differ from evaluation in pots and the field under similar salinity conditions. A barley study showed that genotypic differences in Na^+ and Cl^- contents were much less associated with salt tolerance in hydroponics than in pot and field trials (Tavakkoli et al., 2010; Tavakkoli et al., 2012).

In addition, *Miscanthus* is a perennial that sprouts new shoots from rhizomes after dormancy so it is important to also consider the role of the rhizomes under salt stress. For instance, the size of rhizomes was shown to influence salt tolerance of *Miscanthus* × *giganteus* and common reeds (Bart and Hartman, 2003; Plazek et al., 2014). However, it is still unclear whether the rhizomes influence abiotic tolerance in other *Miscanthus* genotypes. Rhizome-propagated *Miscanthus* genotypes had higher winter survival rates than *in vitro* propagated plants but in another other trial, rhizome-propagated plants showed higher winter mortality than *in vitro* propagated plants (Fonteyne et al., 2016b). Comparing *in vitro*-propagated plants and rhizome-based plants can help to understand the rhizome function in withstanding adverse conditions and help us to choose better propagation methods and material for growth under marginal lands.

Salinity affects plant growth and development due to osmotic stress, ionic stress, and nutritional imbalance (Ashraf and Harris, 2004; Munns and Tester, 2008). Osmotic stress affects growth immediately because of the high concentration of ions in the rhizosphere; the ionic stress develops over time due to ion accumulation within the shoots. Oxidative stress is a secondary stress induced by salinity stress that damages the metabolic pathways due to accumulation of reactive oxygen species (ROS) and the key enzymes for regulating ROS levels such as superoxide dismutase, ascorbate peroxidase, catalase, and the various peroxidases need to be actived and protect the damage from free radicals (Moradi and Ismail, 2007). Basically, improving salt tolerance needs to take these stresses into account.

Salinity stress affects all the major metabolic processes including lipid and energy metabolism, photosynthesis, and protein synthesis (Parida and Das, 2005), and leads to reduction in stomatal conductance, chlorophyll content, tiller number, biomass and increased leaf senescence (Hassanein, 1999; Chartzoulakis and Klapaki, 2000; Munns et al., 2006). The altered water status and unbalanced ion homeostasis induce different mechanisms to reduce damage under salt stress. Osmotic tolerance can be achieved by adapting root water uptake properties, plant hydraulics, and by adjusting the plant osmotic potential. Compatible solutes like proline (Khatkar and Kuhad, 2000), glycine betaine (Khan et al., 2000; Wang and Nii, 2000), sugars (Kerepesi and Galiba, 2000), and polyols (Bohnert et al., 1995; Zhifang and Loescher, 2003) facilitate osmotic adjustment or osmotic protection. A shoot Na^+ exclusion mechanism can exclude surplus sodium ions from the shoot to avoid toxic ion concentrations in shoots. Bread wheat has a low rate of Na^+ transport to the shoot and maintains a high ratio of K^+/Na^+ in the leaves, which contributes to salt tolerance. However, durum wheat is more salt-sensitive due to its poorer ability to exclude Na^+ from the shoot (Gorham et al., 1990). Members of the high-affinity K^+ transporters (HKT) shown to play a role in shoot Na^+ exclusion take Na^+ from the xylem and play an important role in controlling Na^+ concentrations in the shoots of wheat, rice, and sorghum (Conde et al., 2011; Munns et al., 2012; Platten et al., 2013; Wang et al., 2014). A Na^+ inclusion mechanism can compartmentalize Na^+ in cellular and intracellular organelles like the vacuoles (Adams et al., 1992), providing tissue tolerance. Tonoplastic Na^+/H^+ antiporters (NHX) in plants are involved in regulation of ion concentrations and cytosolic pH (Bassil et al., 2012). For instance, maize *ZmNHX*1 and *ZmNHX*6 were highly expressed in the root to maintain high ion concentrations in roots but low ion contents in shoots of salt tolerant maize (Zörb et al., 2005).

Similar to drought stress, salt stress induces decreases in protein or chlorophyll contents and increases in membrane permeability, leading to premature senescence (Munné-Bosch et al., 2001), (Lutts et al., 1996). Leaf senescence is further enhanced under saline conditions because of the accumulation of Na^+ or Cl^- ions, with salt sensitive genotypes showing enhanced senescence and higher ion concentrations in the leaves than salt-tolerant genotypes (Husain et al., 2003a; Munns et al., 2006). Typically, the degree of senescence in mature leaves reflects ion-specific toxicity due to high levels of Na^+ concentrations and low tissue tolerance to Na^+ (Munns and James, 2003). Leaf senescence can therefore be used as a selectable trait in high-thought screening of salt tolerant genotypes (Rajendran et al., 2009; Hairmansis et al., 2014).

To gain additional insight in *Miscanthus* salinity tolerance and the contributing factors and mechanisms, we evaluated 11 genotypes with high salt tolerance or high yield in the hydroponics evaluation (Chen et al., 2017) in a pot experiment under different salinity levels, and *Miscanthus* × *giganteus* as the reference genotype. Two sets of plants were used: plants started from rhizomes, and plants started from *in vitro* culture. Our results showed that rhizomes-started plants showed less senescence than *in vitro*-started plants under salt stress, indicating that rhizomes may support salt tolerance, and that *M. sinensis* genotype OPM-56 may be a high potential resource for growth on saline soils and as a starting material for improvement of salt tolerance of *Miscanthus*.

2 Materials and Methods

2.1 Plant materials

The twelve genotypes of *Miscanthus* used for evaluation are listed in Table 1. This set consisted of 10 *M. sinensis* and 2 hybrids (OPM-9: *Miscanthus* × *giganteus*, OPM-7: *M. sacchariflorus* × *M. sinensis*). Two sources of materials were used for each genotype. One set of the 12 genotypes was propagated from young seedlings following *in vitro* culture and further propagation on hydroponics, and finally transplanted to pots containing vermiculite. For the second set, rhizomes were collected at ILVO (Institute for Agricultural and Fisheries Research ILVO), cut to similar sizes and put in trays with vermiculite for three weeks. Rhizomes with similar-sized tillers per genotype were selected as starting materials and transplanted to pots with vermiculite.

2.2 Pot experiment

Three levels of salt stress were applied to the two different materials (rhizomes-started (R) seedlings and *in vitro* propagated (IV) seedlings without rhizomes): control (0.5X Hoagland solution without additional NaCl), medium salinity with 150 mM NaCl added, and severe salinity with 250 mM NaCl added to the 0.5X Hoagland solution. The seedlings were transplanted to pots and allowed to acclimate and grow for 3 weeks in greenhouse till the 7 leaves-stage for rhizome-started plants and around 10 leaves for *in vitro*-started plants, after which 150 mM or 250 mM NaCl was added to pots in daily steps of 50 mM NaCl. Experiments were conducted from 27th April to15th June 2015. Vermiculite was used as a soil medium to avoid accumulation of ions in the root environment during the experiment and to support a stable salinity stress level. The experiment had a RCBD design with four plants per genotype per treatment. The salt treatment was continued for 6 weeks, after which the plants were harvested. The average day/night temperatures were set at 25/18 °C, and the photoperiod regime was 16 hours light and 8 hours dark. Greenhouse environmental humidity was controlled at 70%. Additional lighting (100 Wm^{-2}) was used if the incoming shortwave radiation was below 200 Wm^{-2}.

Table 1. 12 genotypes screened for salt tolerance

Genotype	Supplier	Genotype
OPM-7	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)
OPM-9	IBERS	Hybrid (*Miscanthus* × *giganteus*)
OPM-31	IBERS	*M. sinensis*
OPM-37	WUR	*M. sinensis*
OPM-42	WUR	*M. sinensis*
OPM-48	WUR	*M. sinensis*
OPM-49	WUR	*M. sinensis*
OPM-56	WUR	*M. sinensis*
OPM-67	WUR	*M. sinensis*
OPM-71	WUR	*M. sinensis*
OPM-76	WUR	*M. sinensis*
OPM-86	WUR	*M. sinensis*

IBERS: Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, UK.
WUR: Wageningen University & Research, the Netherlands

2.3 Measured traits

During the experiment, data were collected with respect to plant height, tiller number and senescence for all plants grown under control and saline conditions. Plant height was measured from the base of the plant to the tip of leaf with a ruler weekly during the experiment. Tiller number was measured at several time points, and senescence was visually assessed during the experiment. Leaf senescence scale was expressed as estimated percentage of senesced leaf area (1 =no senescence, 3 =senesced area 1-30%, 5 =senesced area 30-60%, 7 =senesced area 60-90%, 9 =senesced area > 90%) and was assessed after 2 weeks and 6 weeks of salt treatment. At harvest, all plants were separated into leaves, stems, rhizomes, and roots. Plant shoot fresh weight (SFW) was measured immediately at harvest. Separated plant parts were dried in a forced-air oven at 70°C for 2 days, and shoot dry weight (SDW), rhizome dry weight (RhDW) and root dry weight (RDW) were measured.

2.4 Ion chromatography

For determination of the ion contents in the leaves, stems and roots, four replicated plants of each genotype were separately ground to fine powder using a hammer mill with 1 mm sieve following the protocol described by Nguyen et al. (2013). Dry powders were ashed at 575°C for 5 hours. Ash samples were dissolved by shaking for 30 minutes in 1 ml 3M formic acid at 99°C and then diluted with 9 ml MiliQ water. The samples were shaken again at 80°C for another 30 minutes. A final 500x dilution was subsequently prepared by mixing 0.2 ml sample solution with 9.8ml MiliQ to assess the Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}content of each root and leaf sample using an Ion Chromatography (IC) system (850 Professional, Metrohm, Switzerland).

2.5 Statistical analysis of phenotypic data

Analysis of variance was done to examine the effect of treatments, genotypes and the genotype-by-treatment interactions for the measured parameters. The senescence of each genotype from different resources in control and saline conditions were compared by student's T-test. Significance of the differences of genotypic means in different treatment groups was determined by Fisher's test. Pearson correlation coefficients (r) between all the parameters were calculated. All statistical analyses were performed using the statistical software package Genstat 15th edition (VSN International Hemel Hempstead, UK).

3 Results

3.1 Growth responses of two types of material to different salinity levels

The developmental stage of the two types of materials was slightly different at the start of the salt treatment. The rhizome-derived plants on average were shorter and had less leaves than *in vitro*-started plants. Therefore, comparison of growth traits between the rhizome-derived plants and *in vitro*-started plants should be done with care. The effects of salinity however can be reliably assessed by comparing the plants under control conditions and salt stress conditions. After 6 weeks of salt treatment, plant height of *in vitro*-started plants had decreased significantly at 150 mM NaCl ($p = 0.005$) and 250 mM NaCl ($p < 0.001$) compared to control conditions, but the plant height of rhizome-based plants had only significantly decreased at 250 mM NaCl ($p = 0.002$) compared with 0 mM NaCl (Figure 1a). The average salt-induced reduction of height of *in vitro*-started plants was 9% at 150 mM NaCl and 22% at 250 mM NaCl, and for the rhizome-derived plants 4% at 150 mM NaCl and 15% at 250 mM NaCl. The rhizome-derived plants produced less biomass of both shoots and roots than *in vitro*-started plants in all treatments (Figure 1b and 1c). The shoot dry weight of the *in vitro*-started plants only significantly changed at 250 mM NaCl (p <0.001) compared with 0 and 150 mM NaCl but rhizome-based plants showed a significant reduction at 150 mM NaCl (p =0.003) and at 250 mM (p <0.001) compared with control conditions (Figure 1b). Shoot dry weight was reduced at 150 mM NaCl by 13% and 23% for *in vitro*-started plants and rhizome-derived plants, respectively, and by 48% for *in vitro*-started plans and 44% for rhizome-derived plants at 250 mM NaCl (Figure 1b). The root dry weight of the two types of materials did not change significantly between 0 and 150 mM NaCl, but it showed a significantly decrease in both rhizomes-based plants (p <0.001) and *in vitro*-started plants (p <0.001) at 250 mM NaCl compared with 0 mM NaCl (Figure 1c). The average root dry weight of *in vitro*-started plants decreased from 7.77 g at 0 mM NaCl to 3.73 g at 250 mM NaCl and in rhizomes-based plants from 3.79 g at 0 mM NaCl to 1.84 g at 250 mM NaCl.

The average number of tillers in the *in vitro*-started plants was reduced from 7.2 at 0 mM NaCl to 5.3 at 150 mM NaCl (p =0.001) and to 3.7 at 250 mM NaCl (p <0.001). Tiller number in rhizome-based plants however was only slightly reduced at 150 mM NaCl, but significantly decreased from 4.1 under control conditions to 2.4 at 250 mM NaCl (p =0.002) (Figure 1d). *In vitro*-started plants produced more tillers than rhizome-based ones and the salt-induced reduction of the former (49%) was higher than the latter (41%) at 250 mM NaCl. After 2 weeks of salt treatment, the senescence score of the two types of materials significantly increased compared to control conditions but there was no significant difference between 150 mM NaCl and 250 mM NaCl (Figure 1e). Senescence of the *in vitro*-started plants increased significantly from 1.98 at 0 mM NaCl to 3.28 at 150 mM NaCl (p =0.004) and 3.45 at 250 mM NaCl (p<0.001). Similarly, senescence in rhizome-based plants was significantly increased from 1.67 to 2.38 at 150 mM NaCl (p <0.001) and 2.49 at 250 mM NaCl (p <0.001) (Figure 1e). After 6 weeks of salt stress, senescence of the *in vitro*-started plants was significantly increased from 2.83 under control conditions to 5.15 at 150 mM NaCl (p <0.001) and 6.08 at 250 mM NaCl (p <0.001) and in rhizome-based plants from 2.53 under control conditions to 4.12 at 150 mM NaCl (p <0.001) and 4.85 at 250 mM NaCl (p <0.001) (Figure 1f). The average senescence of *in vitro*-started plants was higher than rhizome-based plants at both 150 and 250 mM NaCl, which may indicate that rhizomes have the ability to delay salt stress-induced senescence.

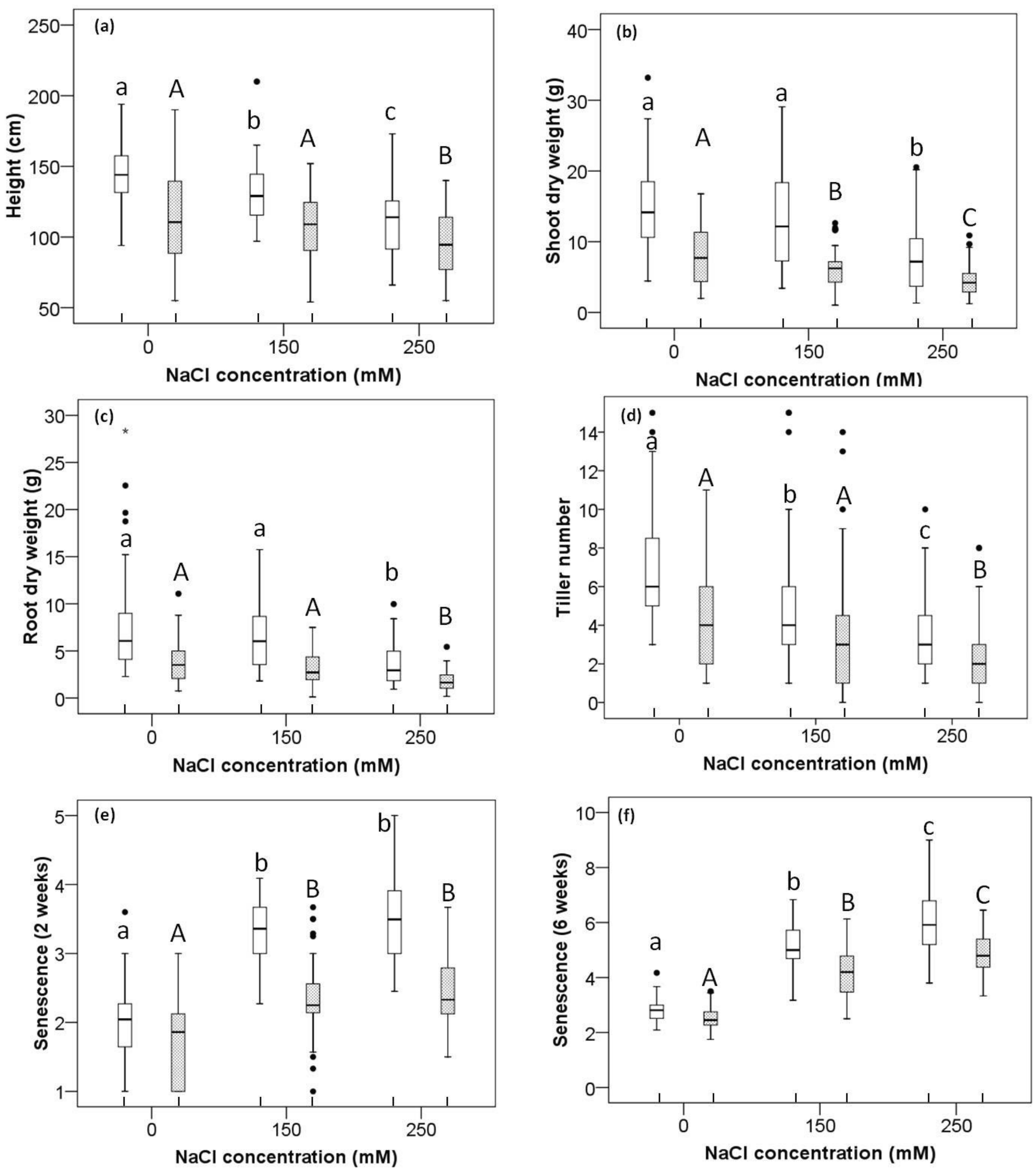

Figure 1. Box plots of growth trait data of *Miscanthus* from *in vitro*-started plants (white bar) and rhizome-derived plants (gray bar) at 0, 150 and 250 mM NaCl. Height (a), shoot dry weight (b), root dry weight (c), tiller number (d), senescence at 2 weeks (e), senescence at 6 weeks (f). Box edges show upper and lower quartile and the median is shown in the middle of the box. Mild outliers are shown as dots and extreme outliers are shown as stars. Different letters indicate significant differences between salt treatments for each type of materials (LSD $_{0.05}$ test).

3.2 Na^+, Cl^-, and K^+ contents

The average leaf Na^+ contents in both rhizome and *in vitro*-derived material was significantly increased (P <0.001) at 250 mM NaCl compared to control conditions, but not significantly different between 0 and 150 mM NaCl (Figure 2a). The average Na^+ contents in leaves of *in vitro*-started plants increased from 2.78 mg/g DW under control conditions to 11.41 mg/g DW at 250 mM NaCl and from 4.05 mg/g DW at 0 mM NaCl to 13.51 mg/g DW at 250 mM NaCl in rhizome-derived plants. The average leaf Cl^- contents was significantly increased at 150 mM NaCl in *in vitro*-started plants, from 3.19 mg/g DW to 6.87 mg/g DW (P =0.03) at 150 mM NaCl and to 20.69 mg/g DW (P <0.001) at 250 mM NaCl. Similarly, the leaf Cl^- contents of rhizome-derived plants showed a significantly increase with 7.54 mg/g DW (P <0.009) and 23.71 mg/g DW (P <0.001) at 150 and 250 mM NaCl, respectively compared with 2.89 mg/g DW under control conditions (Figure 2b). Root Na^+ contents in the *in vitro*-started plants significantly increased from 3.29 mg/g DW under control conditions to 16.16 mg/g DW (P <0.001) and 17.82 mg/g DW (P <0.001) at 150 and 250 mM NaCl, respectively (Figure 2c). Root Na^+ contents in the rhizome-based plants significantly increased from 3.7 mg/g DW under control condition to 17.48 mg/g DW (P <0.001) at 150 mM NaCl and 21.25 mg/g DW (P <0.001) at 250 mM NaCl. Root Cl^- contents responded to the salt treatments similar to root Na^+ contents. The concentrations were already increased in roots at 150 mM NaCl while in leaves these were not significantly affected at this mild stress level, indicating that the *Miscanthus* genotypes utilized an ion shoot exclusion mechanism under these conditions (Figure 2a and 2c).

The average leaf K^+ content of *in vitro*-started plants at 150 mM NaCl was significantly lower (6.40 mg/g DW) than that under control conditions (8.83 mg/g DW). Remarkably, the leaf K^+ content was increased to even higher levels than control at 250 mM NaCl (11.27 mg/g DW) (Figure 2e). Similarly, the average K^+ contents in leaves of rhizome-derived plants decreased from 10.25 mg/g DW at 0 mM NaCl to 5.87 mg/g DW at 150 mM NaCl and increased to 11.66 mg/g DW at 250 mM NaCl. The K^+ contents in root however were decreased at both salt levels and in both types of material compared with control conditions (Figure 2f), pointing to an active mechanism to increase leaf K^+ levels at higher salinity, thus maintaining a more favourable K^+/Na^+ ratio. For the leaf K^+/Na^+ ratio, there was no significant difference between control and 150 mM NaCl while it showed a significant decrease in rhizome-derived plants (P <0.001) and *in vitro*-started plants (P =0.001) at 250 mM NaCl compared with control conditions (Figure 2g). However, the root K^+/Na^+ ratio in two types of materials showed a significant decrease at 150 mM and 250 mM NaCl compared with control conditions (Figure 2h).

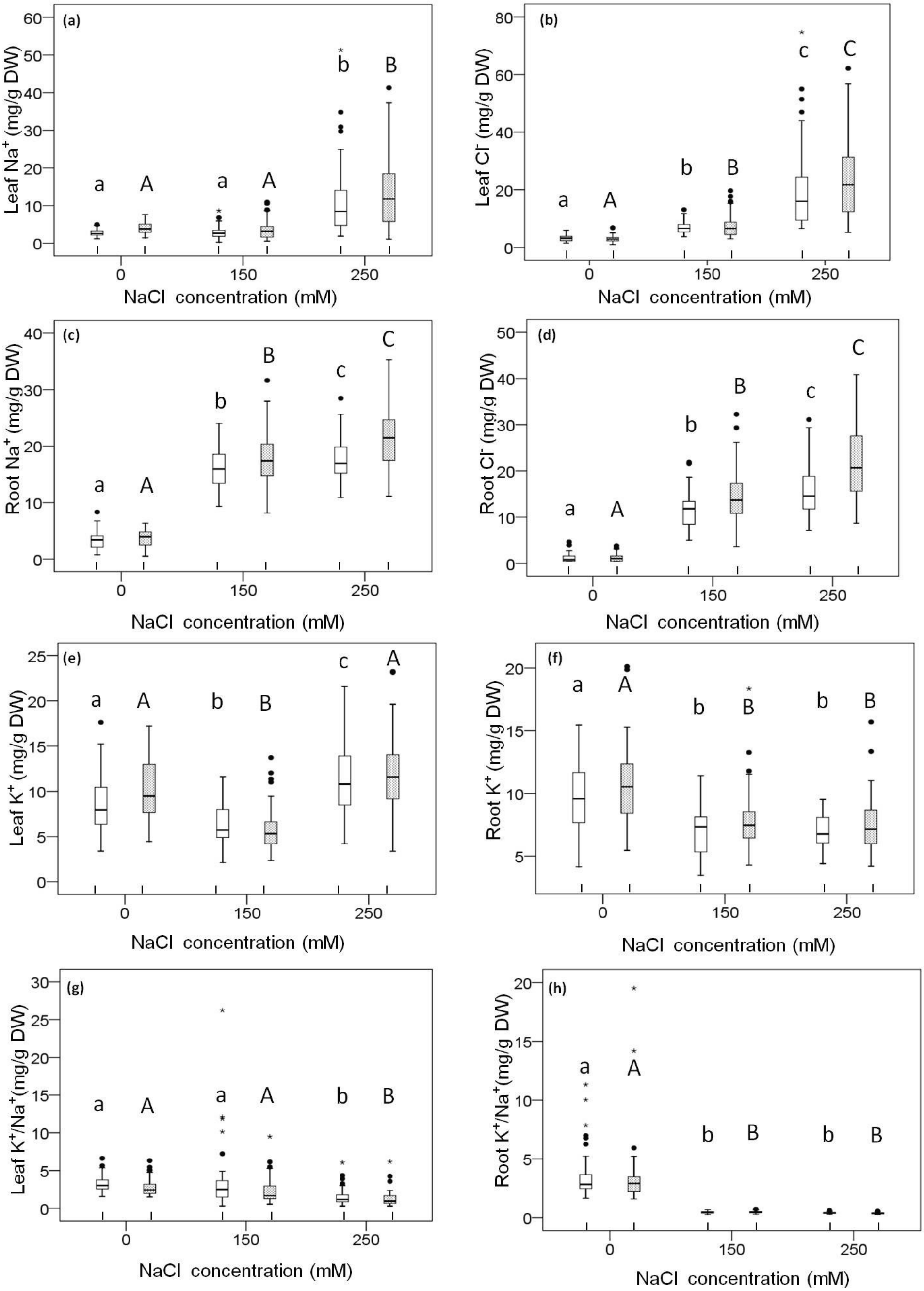

Figure 2. Box plots of ion contents of of *Miscanthus* of *in vitro*-started plants (white bar) and rhizomes (gray bar) at 0, 150 and 250 mM NaCl. Na^+ in leaves (a), Cl^- in leaves (b), Na^+ in roots (c), Cl^- in roots (d), K^+ in leaves (e), K^+ in roots (f), K^+/Na^+ in leaves (g) and roots (h). Box edges show upper and lower quartile and the median is shown in the middle of the box. Mild outliers are shown as dots and extreme outliers are shown as stars. Different letters indicate significant differences between salt treatments for each type of materials (LSD $_{0.05}$ test).

3.3 Shoot dry weight and salt tolerance

Salt tolerance was assessed as the percentage of shoot dry biomass under saline relative to control conditions. The average salt tolerance of the 12 genotypes derived from *in vitro* plants was 88% at 150 mM NaCl and 51% at 250 mM NaCl, and 79% and 57% in rhizome-based plants at 150 and 250 mM NaCl, respectively. Biomass production of *Miscanthus* genotypes was thus already mildly affected at 150 mM NaCl but the 250 mM NaCl treatment caused on average nearly 50% reduction in shoot dry weight in both materials. At the high salinity level, the genotypes without rhizomes showed large variation for salt tolerance, from 26% in genotype OPM-86 to 89% in genotype OPM-9 and the rhizome-based plants varied from 38% in genotype OPM-9 to 88% in genotype OPM-67 (Figure 3a and b). Averaged over the two types of materials, the genotypes OPM-48, 56, and 67 were relatively more salt tolerant (almost 70%) than genotype OPM-9 (*Miscanthus* × *giganteus*) with 63% salt tolerance at 250 mM NaCl. Genotype OPM-56 had the highest biomass in both control (15.77 g)

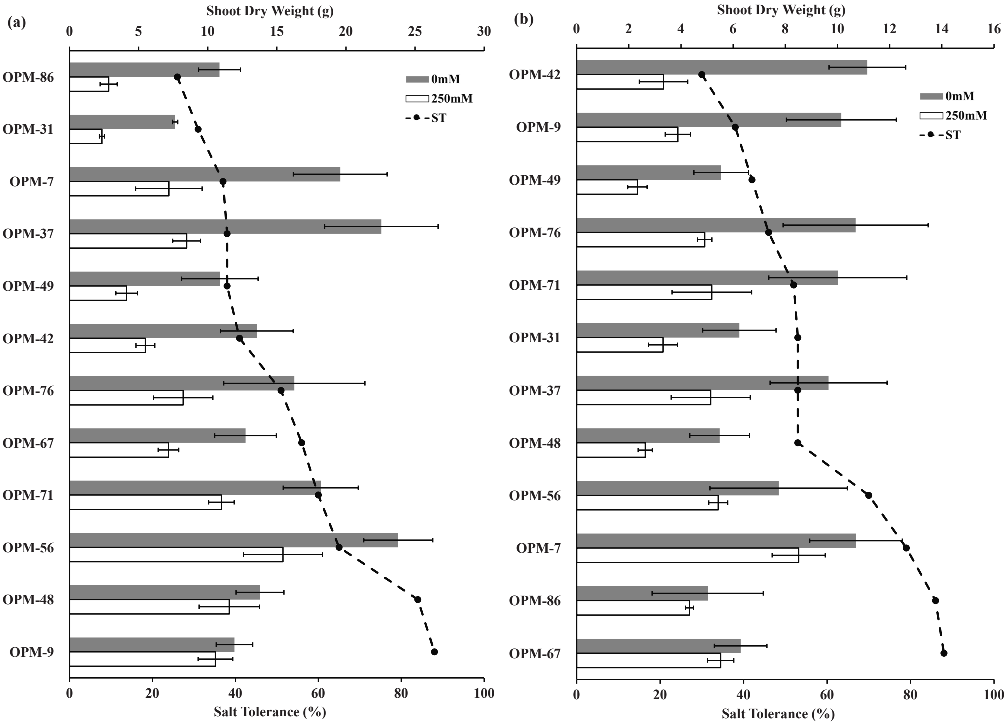

Figure 3. Twelve genotypes with two types of materials: *In vitro*-started plants (a) and rhizome-derived plants (b). Shoot dry weight (bars) and salt tolerance (line-scatter plot, calculated as the ratio of shoot dry weight under salt stress and shoot dry weight under control conditions) of 12 *Miscanthus* genotypes at 0 mM NaCl (gray bars) and 250 mM NaCl (white bars) for 6 weeks. Error bars indicate standard error (n =4).

and salt stress (10.44 g) while OPM-9 had lower yields under both conditions: 11.04 g at 0 mM NaCl and 7.22 g at 250 mM NaCl. Genotype OPM-56 is therefore a candidate for production of biomass under salt conditions; it has not only the highest yield under salt stress but was also relatively salt tolerant on average (67%). While salt tolerance of some genotypes was quite different between rhizome- and *in vitro*-derived plants, salt tolerance of OPM-56 of these two different materials was comparable (70% vs 65%).

3.4 Effects of salinity on growth traits in different genotypes

The high salinity level (250 mM NaCl) significantly reduced root dry weight of 3 genotypes (OPM-9, 42 and 49) of rhizome-started plants and 5 genotypes (OPM-9, 31, 37, 42 and 67) of *in vitro*-started plants (Figure 4). No significant differences between 0 and 250 mM NaCl were detected when comparing shoot dry weight /root dry weight ratio in both two types of materials (Figure 5). The rhizome dry weight of 11 genotypes showed no significant differences at three different salt levels, while the rhizome dry weight of genotype OPM-42 significantly decreased at 150 and 250 mM NaCl compared with control conditions (Supplementary Table 1).

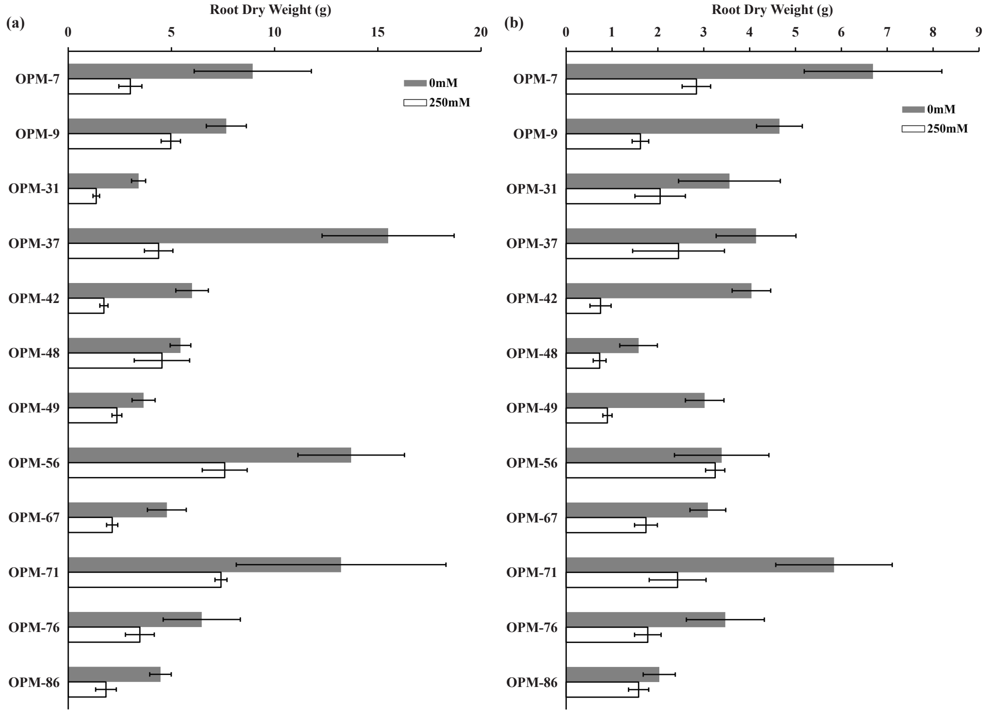

Figure 4. The root dry weight of 12 *Miscanthus* genotypes at 0 mM NaCl (gray bars) and 250 mM NaCl (white bars) after 6 weeks. Twelve genotypes with two types of materials: *In vitro*-started plants (a) and rhizome-derived plants (b). Error bars indicate standard error (n =4).

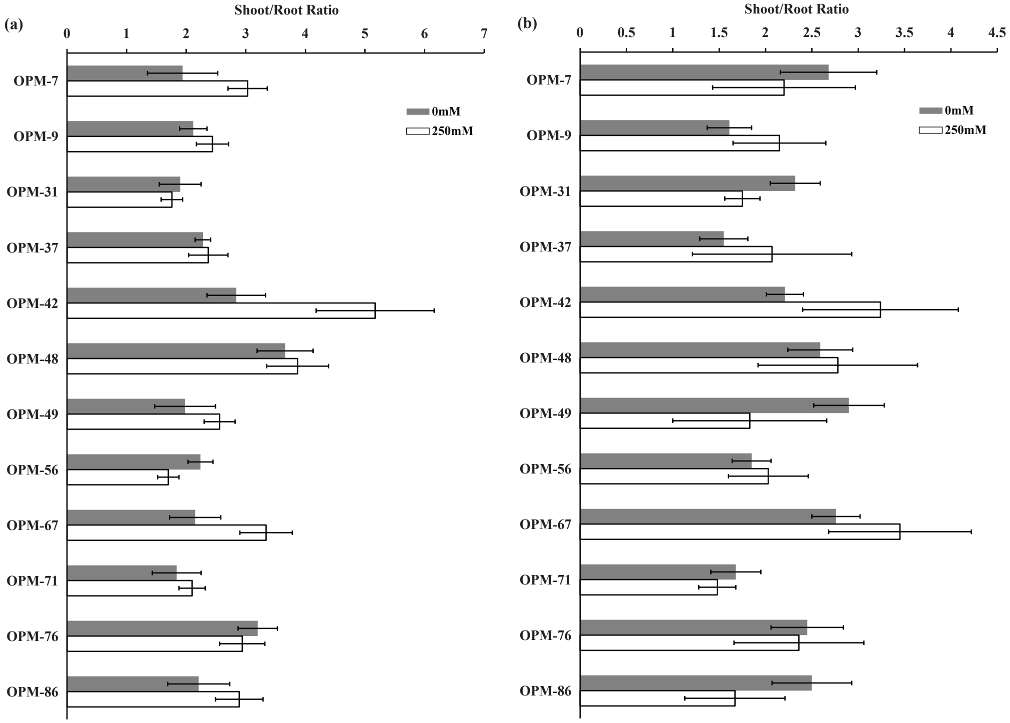

Figure 5. The shoot/root ratio of twelve genotypes with two types of materials: *In vitro*-started plants (a) and rhizome-derived plants (b). Twelve *Miscanthus* genotypes at 0 mM NaCl (gray bars) and 250 mM NaCl (white bars) for 6 weeks. Error bars represent standard error (n =4).

Differences between plants with rhizomes and *in vitro*-started plants were also observed for senescence. Salt-stressed plants were more senesced than control plants already after 2 weeks of salt stress, and rhizome-derived plants had less senescence then *in vitro*-started plants at 150 mM NaCl and 250 mM NaCl (Supplementary Table 2). Senescence increased with time and salt concentration; after 6 weeks of salinity stress, the average senescence of plants without rhizomes at 250 mM NaCl was higher than plants with rhizomes (6.1 vs 4.9). *In vitro*-started plants of genotypes OPM-9 and OPM-71 had the highest senescence levels (Table 2). Genotype OPM-86 started from rhizomes not only showed lower senescence than *in vitro* plants at 150 mM and 250 mM NaCl but also had green flag leaves while the flag leaves of OPM-86 started from *in vitro* plants were senescing (Supplementary Figure 1).

Table 2. Senescence of twelve genotypes with rhizome-derived plants (R) and *in vitro*-started plants (IV) after 6-week treatment at three different salt levels.

Genotype	0 mM NaCl					150 mM NaCl					250 mM NaCl				
	R		IV		Sig.	R		IV		Sig.	R		IV		Sig.
	Mean	±SE	Mean	±SE		Mean	±SE	Mean	±SE		Mean	±SE	Mean	±SE	
OPM-07	2.44	0.11	2.75	0.10		4.18	0.20	4.86	0.11	*	4.67	0.22	6.1	0.45	*
OPM-09	2.65	0.18	3.22	0.26		2.93	0.20	5.27	0.31	***	4.88	0.16	7.28	0.78	*
OPM-31	2.66	0.12	2.57	0.04		4.11	0.33	4.99	0.24		5.09	0.33	5.65	0.36	
OPM-37	2.74	0.25	2.9	0.12		4.22	0.34	5.29	0.17	*	5.44	0.04	5.78	0.25	
OPM-42	2.28	0.04	2.64	0.15		3.61	0.27	4.95	0.09	**	4.32	0.30	5.16	0.13	*
OPM-48	1.89	0.07	2.66	0.11	***	3.16	0.23	3.46	0.18		3.85	0.31	4.28	0.21	
OPM-49	2.38	0.11	2.7	0.34		4.6	0.14	5.64	0.47		4.96	0.27	6.49	0.17	**
OPM-56	2.86	0.19	2.96	0.11		4.51	0.45	4.94	0.23		4.4	0.18	5.91	0.24	**
OPM-67	2.52	0.12	2.58	0.07		4.65	0.22	4.76	0.28		5.36	0.06	5.16	0.13	
OPM-71	2.44	0.08	3.34	0.28	*	4.45	0.27	5.41	0.37		5.05	0.50	7.52	0.59	*
OPM-76	2.85	0.23	3.19	0.13		4.89	0.57	6.14	0.25		5.92	0.23	6.69	0.32	
OPM-86	2.63	0.24	2.51	0.08		4.22	0.39	6.14	0.27	**	4.38	0.16	6.93	0.13	***

*;**;***: significant at $P<0.05$; 0.01; 0.001 respectively

3.5 Genotypic variation of ion contents

Leaf Na^+ contents of the 12 genotypes were comparable at 0 and 150 mM NaCl, while there was a large variation at 250 mM NaCl, ranging from 2.55 mg/g DW in genotype OPM-48 to 30.14 mg/g DW in genotype OPM-9 for *in vitro*-started plants and from 3.58 mg/g DW in genotype OPM-56 to 25.01 mg/g DW in genotype OPM-71 of rhizome-based plants (Figure 6). Similarly, genotypic variation in leaf Cl^- contents of the *in vitro*-started plants ranged from 8.47 mg/g DW in genotype OPM-48 to 47.35 mg/g DW in genotype OPM-9, and from 6.93 mg/g DW in genotype OPM-56 to 40.46 mg/g DW in genotype OPM-71 for rhizome-based plants at 250 mM NaCl. Detailed ion data including leaf Na^+ Cl^- and K^+ are given in Supplementary Table 3, 4 and 5. Several *Miscanthus* genotypes without rhizomes had higher K^+ concentrations at 250 mM NaCl than at 150 mM NaCl (OPM-49, 71, and 86). In rhizome-based plants, genotypes OPM-9, 37, 49 and 71 had higher K^+ concentrations at 250 mM NaCl than 150 mM NaCl (Supplementary Table 5). At 250 mM NaCl, the leaf K^+ contents of *in vitro*-started OPM-9 and OPM-71 plants were higher than 15 mg/g DW while that of genotype OPM-56 and OPM-67 was lower than 7 mg/g DW; the leaf K^+ contents in genotype OPM-37, 49 and 71 of rhizome-based plants was higher than 15 mg/g DW but genotype OPM-56 had lower values (4.9 mg/g DW) (Supplementary Table 5).

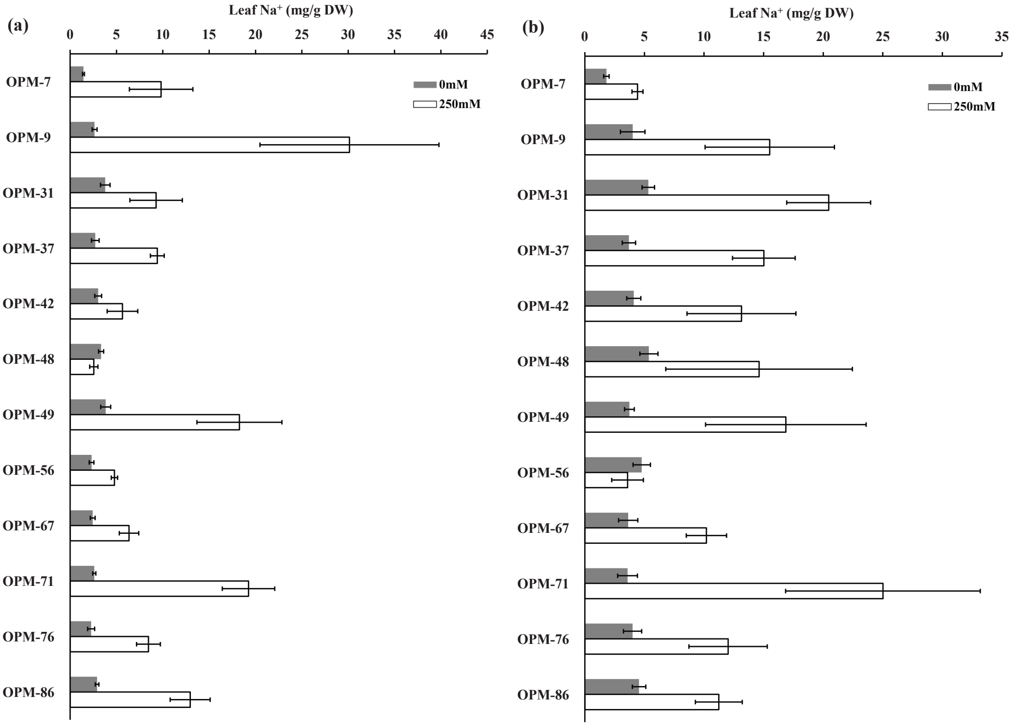

Figure 6. Leaf Na^+ contents of the 12 genotypes of two types of materials: *In vitro*-started plants (a) and rhizome-derived plants (b). Twelve *Miscanthus* genotypes at 0 mM NaCl (gray bars) and 250 mM NaCl (white bars) for 6-week treatment. Error bars represent standard error (n =4).

Since stems are the major part of *Miscanthus* for combustion, low stem ion contents under salt stress may contribute to combustion quality. Genotype OPM-56 had the lowest total ion content (Na^+, Cl^- and K^+ together) of the *in vitro*-started plants (16.5 mg/g DW) and this was relatively low in rhizome-derived plants (21.52 mg/g DW), while the commercial genotype OPM-9 (*Miscanthus* × *giganteus*) had the highest of the *in vitro*-started plants (93.23 mg/g DW) and relatively high ion contents with 60.54 mg/g DW in rhizome-based plants (Supplementary Table 3, 4 and 5). Genotype OPM-56 had lowest stem Na^+ (Figure 7) and Cl^- ion contents for both materials, while OPM-9 was high for both these ions. Furthermore, genotype OPM-56 had relatively low K^+ contents with 7.26 mg/g DW in rhizome-derived plants and 5.84 mg/g DW of *in vitro*-started plants while genotype OPM-9 contained relatively high K^+ contents with 17.76 mg/g DW and 15.83 mg/g DW of *in vitro*-started and rhizome-based plants, respectively (Supplementary Table 5).

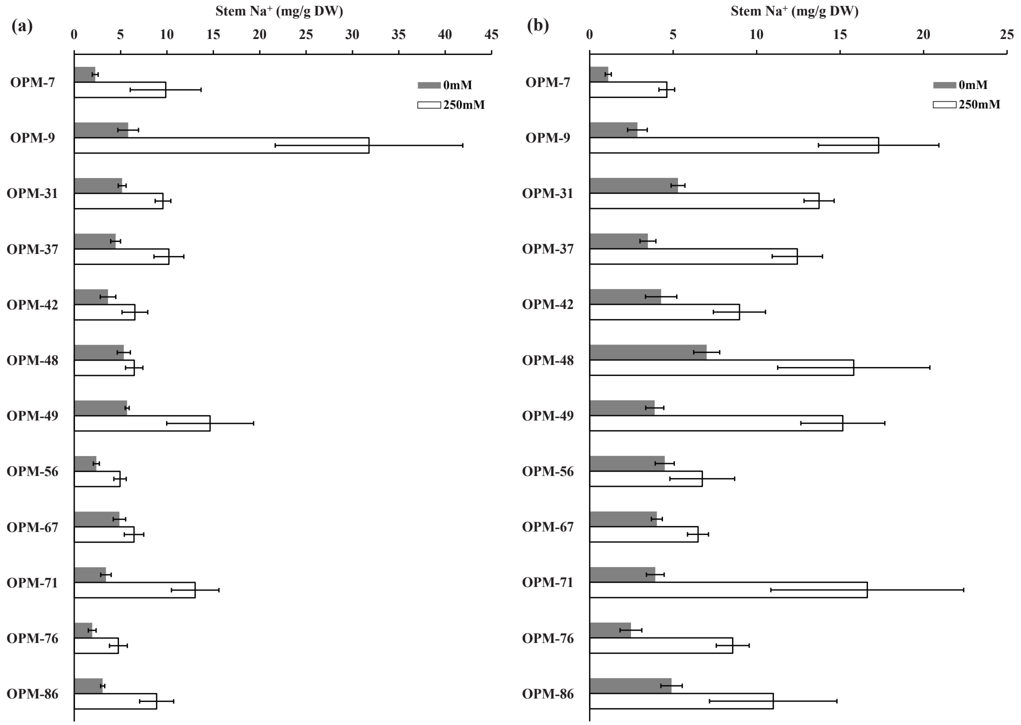

Figure 7. The stem Na^+ contents of the 12 genotypes of two types of materials: *In vitro*-started plants (a) and rhizome-derived plants (b). Twelve *Miscanthus* genotypes at 0 mM NaCl (gray bars) and 250 mM NaCl (white bars) for 6-week treatment. Error bars indicate standard error (n =4).

3.6 Correlation Analysis

The results presented above indicate that rhizome-derived and *in vitro*-started plants have distinctly different responses to salinity. We further investigated this different behavior by calculating Pearson correlations between the physiological traits and ion contents for the two types of materials at 250 mM NaCl (Table 3). Shoot dry weight was positively correlated with height, stem height, root dry weight, and tiller number in both types of material at 250 mM NaCl. Similarly, the stem dry weight was positively linked to height, stem height, root dry weight, and tiller number in the *in vitro*-started plants at 250 mM NaCl. A highly significant positive correlation between Cl^- and Na^+ contents in leaves, stems and roots was found for both two materials at 250 mM NaCl ($P < 0.001$ and $r > 0.94$). Under salt stress, there was a high correlation between Cl^- and K^+ contents of rhizome-derived as well as *in vitro*-derived plants in leaves, stems and roots. The correlation between K^+ and Na^+ contents of rhizome-derived plants was as high as the *in vitro*-started plants in leaves, stems and roots. However, remarkable differences were found for the correlations of ion contents with growth traits between rhizome-derived and *in vitro*-started plants. The shoot dry weight of the rhizome-based plants was strongly positively related to the K^+/Na^+ ratio in leaves ($r = 0.82$) but this correlation was absent in the *in vitro*-started plants at 250 mM NaCl. There was a significant and positive correlation of tiller number with K^+/Na^+ ratio in leaves (r =0.82) and a negative relationship with Na^+ (r =-0.78) and Cl^- (r =-0.77) in rhizome-derived plants, but the tiller number of *in vitro*-started plants had no significant relationship with ion data in leaves. On the other hand, senescence in the *in vitro*-started plants positively correlated with Na^+ (r =0.79), Cl^- (r =0.80) and K^+ (r =0.76) contents and negatively correlated with the K^+/Na^+ ratio (r =-0.71), while there was no significant correlation between senescence and these ion traits in the rhizome-based plants. The leaf ion contents did not show significant differences between *in vitro*-started plants and rhizome-derived plants, but there was only a significant positive correlation between senescence and leaf ion content (Na^+ and Cl^-) in the *in vitro*-started plants (Figure 8 a and b), indicating that the rhizomes may interfere with ion toxicity induced leaf senescence.

Table 3. Person's correlation coefficients between the traits of *in vitro*-started plants (left lower triangle) and rhizome-derived plants (right upper triangle) at 250 mM NaCl.

	SDW	SWF	LDW	StDW	RDW	Height	StH	Tiller No.	ST	SEN	Leaf Na^+	Stem Na^+	Root Na^+	Leaf Cl^-	Stem Cl^-	Root Cl^-	Leaf K^+	Stem K^+	Root K^+	Leaf K^+/Na^+	Stem K^+/Na^+	Root K^+/Na^+
SDW	0.23	0.98	0.91	0.95	0.77	0.44	0.72	0.76	0.54	0.22	-0.54	-0.67	-0.65	-0.46	-0.77	-0.68	-0.20	-0.71	-0.23	0.82	0.33	0.62
SWF	0.99	0.05	0.91	0.91	0.74	0.39	0.63	0.69	0.65	0.24	-0.54	-0.66	-0.62	-0.45	-0.74	-0.66	-0.17	-0.67	-0.19	0.80	0.34	0.62
LDW	0.95	0.95	-0.15	0.74	0.62	0.08	0.46	0.64	0.51	0.15	-0.42	-0.66	-0.50	-0.38	-0.64	-0.58	-0.14	-0.45	-0.25	0.75	0.57	0.44
StDW	0.97	0.95	0.83	0.46	0.78	0.66	0.82	0.76	0.50	0.25	-0.56	-0.60	-0.69	-0.47	-0.77	-0.68	-0.22	-0.82	-0.19	0.77	0.12	0.68
RDW	0.90	0.91	0.87	0.86	0.52	0.57	0.78	0.64	0.39	0.25	-0.33	-0.40	-0.50	-0.27	-0.57	-0.55	-0.16	-0.63	-0.17	0.58	-0.12	0.43
Height	0.66	0.66	0.51	0.73	0.46	0.55	0.73	0.38	0.20	0.24	-0.41	-0.15	-0.33	-0.29	-0.43	-0.23	-0.11	-0.75	0.19	0.45	-0.49	0.60
StH	0.49	0.47	0.24	0.66	0.48	0.65	0.82	0.70	0.15	0.35	-0.51	-0.49	-0.64	-0.38	-0.69	-0.62	-0.13	-0.82	-0.29	0.67	-0.16	0.47
Tiller No.	0.83	0.79	0.67	0.89	0.80	0.46	0.62	0.68	0.31	-0.11	-0.78	-0.77	-0.65	-0.77	-0.82	-0.58	-0.62	-0.79	-0.31	0.82	0.31	0.52
ST	0.76	0.78	0.84	0.63	0.60	0.59	0.01	0.36	-0.26	-0.03	-0.46	-0.51	-0.31	-0.47	-0.56	-0.39	-0.39	-0.46	-0.15	0.38	0.22	0.23
SEN	-0.02	0.02	-0.03	-0.01	0.29	-0.04	0.22	0.11	-0.07	0.23	0.22	-0.08	-0.54	0.36	-0.10	-0.56	0.54	-0.13	-0.45	0.00	-0.05	0.06
Leaf Na^+	-0.04	0.04	0.07	-0.13	0.18	-0.02	-0.13	-0.14	0.23	0.79	0.43	0.82	0.37	0.97	0.86	0.28	0.73	0.83	0.09	-0.75	-0.31	-0.44
Stem Na^+	0.05	0.15	0.18	-0.05	0.16	0.06	-0.24	-0.13	0.41	0.55	0.93	0.60	0.55	0.82	0.94	0.54	0.62	0.70	0.49	-0.67	-0.65	-0.19
Root Na^+	-0.33	-0.25	-0.16	-0.44	-0.34	-0.28	-0.57	-0.46	0.07	-0.07	0.37	0.54	-0.81	0.30	0.61	0.97	0.12	0.63	0.68	-0.52	-0.15	-0.44
Leaf Cl^-	-0.05	0.04	0.04	-0.11	0.19	-0.01	-0.07	-0.14	0.19	0.80	0.99	0.91	0.33	0.59	0.84	0.21	0.88	0.76	0.08	-0.62	-0.36	-0.34
Stem Cl^-	-0.03	0.06	0.11	-0.15	0.11	0.00	-0.31	-0.22	0.37	0.59	0.95	0.98	0.51	0.93	0.57	0.60	0.63	0.89	0.38	-0.71	-0.38	-0.37
Root Cl^-	-0.25	-0.18	-0.10	-0.36	-0.33	-0.15	-0.53	-0.41	0.18	-0.10	0.36	0.55	0.98	0.31	0.52	0.88	0.04	0.55	0.75	-0.48	-0.18	-0.32
Leaf K^+	-0.17	-0.10	-0.12	-0.20	0.12	-0.21	-0.06	-0.19	-0.04	0.76	0.79	0.64	0.13	0.85	0.70	0.09	0.81	0.53	0.07	-0.27	-0.27	-0.10
Stem K^+	-0.36	-0.28	-0.16	-0.49	-0.15	-0.26	-0.54	-0.60	0.13	0.50	0.78	0.73	0.40	0.78	0.83	0.35	0.72	0.61	0.17	-0.72	0.03	-0.60
Root K^+	-0.02	0.08	0.09	-0.11	-0.04	0.06	-0.31	-0.27	0.39	0.24	0.66	0.84	0.70	0.66	0.80	0.71	0.51	0.62	-0.82	-0.11	-0.37	0.35
Leaf K^+/Na^+	0.30	0.27	0.29	0.29	0.09	0.15	-0.09	0.11	0.35	-0.71	-0.61	-0.43	-0.12	-0.57	-0.44	-0.06	-0.31	-0.34	-0.09	-0.16	0.31	0.64
Stem K^+/Na^+	-0.15	-0.23	-0.22	-0.09	-0.31	0.15	0.07	-0.21	-0.13	-0.39	-0.66	-0.73	-0.46	-0.65	-0.66	-0.41	-0.44	-0.27	-0.50	0.41	0.25	-0.14
Root K^+/Na^+	0.37	0.35	0.23	0.45	0.36	0.48	0.54	0.30	0.24	0.34	0.13	0.07	-0.66	0.19	0.07	-0.61	0.33	0.06	0.06	0.08	0.21	0.60

SDW, Shoot dry weigh; SFW, Shoot fresh weight; LDW, Leaf dry weight; StDW, Stem dry weight; RDW, Root dry weight; StH, Stem Height; ST, Salt tolerance; SEN, Senescence.
From light red to dark red, increasingly more positive correlation. From light blue to dark blue, increasingly more negative correlation.

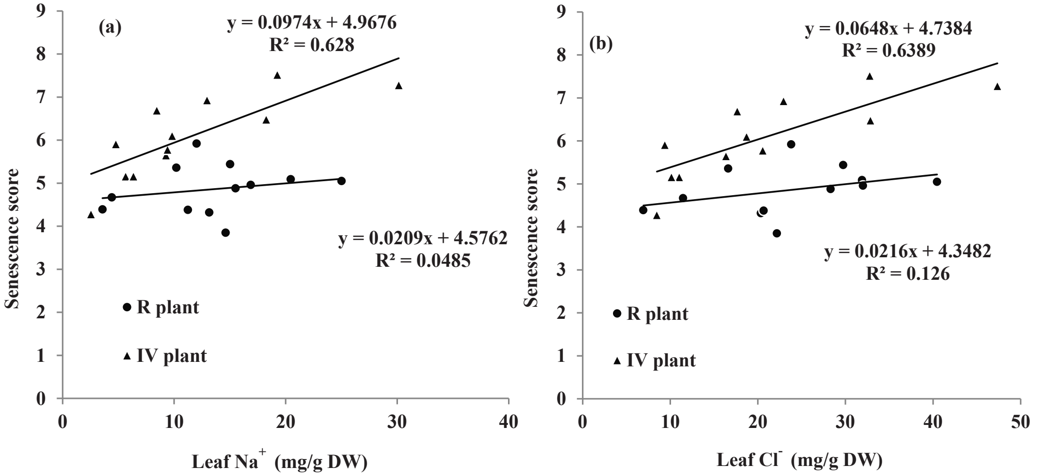

Figure 8. Regression analysis between senescence and leaf Na^+ (a) and Cl^- (b) contents in rhizome-derived plants (R) and *in vitro*-started plants (IV). R^2 =percentage of variance of senescence explained by ion contents.

4 Discussion

Bioenergy crops are an important alternative for fossil fuel and *Miscanthus* has been considered as a potential bioenergy crop in Europe since the 1980s (Brosse et al., 2012; Chung and Kim, 2012). An important breeding goal for bioenergy crops is to achieve economically viable yields in marginal lands and to avoid competition with food crops and food security (Somerville et al., 2010). Therefore, *Miscanthus* is required to be grown on underutilized marginal lands like saline soils to avoid competition with other agricultural lands. Screening novel *Miscanthus* germplasm for the ability to be grown under marginal conditions and evaluating their performance under stress is essential to expand the production area and support an economically feasible biomass. Most studies on stress tolerance of *Miscanthus* use a single genotype, *Miscanthus* × *giganteus*, with rhizome-based plants as starting material (Plazek et al., 2014; Stavridou et al., 2016). We have reported previously on a more extensive evaluation with a large set of genotypes, using *in vitro* plants that were transferred to a hydroponics system (Chen et al., 2017). To further understand salt tolerance mechanisms of *Miscanthus* and the possible role of the rhizome, we evaluated in this chapter the impact of the two types of starting materials (plants with or without rhizome). Our study with twelve *Miscanthus* genotypes at 3 salt levels identified several genotypes with high salt tolerance and low ion contents in both two-type materials at 250 mM NaCl, and also indicates that the rhizome may interfere with ion toxicity in the shoots of the plants.

4.1 Salt severity

Several studies have evaluated salt tolerance of *Miscanthus*, using salt concentrations varying from 0 to 240 mM NaCl applied to *Miscanthus* × *giganteus* with rhizomes (Plazek et al., 2014; Stavridou et al., 2016) and from 0 to 360 mM NaCl for *M. sinensis* plants (Sun et al., 2014). Plazek et al. (2014) reported a significant reduction of shoot dry weight in *Miscanthus* × *giganteus* at 150 mM NaCl (50%), but the root dry weight only had a significant reduction at 200 mM after 12 weeks of treatment when rhizomes were planted directly in perlite with Hoagland solutions supplied with the corresponding NaCl concentrations. Stavridou *et al*. (2016) reported a yield reduction of *Miscanthus* × *giganteus* of 50% at 120 mM NaCl, and significant reduction of root dry weight only at 240 mM NaCl in their experiments using pots containing compost, after 64-day treatment with 0.5X Hoagland solutions supplied with additional NaCl. Sun et al. (2014) found a reduction of shoot dry weight in *M. sinensis* seedlings of 50% in tolerant accessions and 65 % in sensitive accessions at 240 mM NaCl, and around 40 % in tolerant accessions and 50 % in sensitive accessions at 120 mM NaCl. The average reduction of shoot dry weight in our study was only 18% at 150 mM NaCl, and 46% at 250 mM NaCl. The relatively high salt tolerance level of the tested genotypes compared to other studies may at least partly be due to the fact that these genotypes were selected for their high salt tolerance or high yield in a previous salt tolerance study of 70 *Miscanthus* genotypes on a hydroponics system at 150 mM NaCl (Chen et al., 2017). The average salt tolerance of the 12 tested genotypes was higher (53%) than the average of the 70 genotypes (43%) and the yield was also higher (0.87g) than the average biomass (0.77g) of the 70 tested genotypes under salt stress. However, there was no significant correlation in salt tolerance of the selected genotypes between hydroponics and our pot experiments. One possible explanation may be that the the plants were slightly older in the pot experiments than in the hydroponic system. Alternatively, the continuous exposure to salt and the liquid root environment may cause different responses between hydroponics system and pots (Tavakkoli et al., 2012). Pearson correlations of salt tolerance between hydroponics are given in Supplementary Table 6.

In addition to the significant reduction in shoot dry weight, the root dry weight of the 12 genotypes was only significantly reduced (51%) at 250 mM NaCl (Figure 1c). In line with the results from Stavridou et al. (2016) and Plazek et al. (2014), the *Miscanthus* plants seem to only adapt their root biomass at higher levels of salt stress. Moreover, there were significant increases in Na^+ and Cl^- contents of leaves of the 12 genotypes ($P<0.001$) between control and salt treatment ($P <0.001$), but only at the high salt level (250 mM NaCl) (Figure 2a and b). Na^+ and Cl^- contents were already increased in roots at 150 mM NaCl, but the salt-induced increase in leaves was only significant at 250 mM NaCl. This indicates that the evaluated genotypes had exclusion mechanisms that enabled the leaves to maintain a low concentration of Na^+, and be relatively unaffected by by the moderate salt stress at 150 mM NaCl, which is reflected in limited reduction in growth, and hardly any adaptation of the roots in terms of biomass. Maintaining low Na^+ contents in leaves to avoid ion toxicity is a common salt tolerance mechanism in glycophytic plants (Assaha et al., 2017). Generally, the toxicity symptoms of Na^+ occur when leaves accumulate from 3 to 10 mg/g DW (Ayers and Westcot, 1985). The average leaf Na^+ content was 3.2 mg/g in two types of materials at 150 mM NaCl, but it increased to 12.5 mg/g at 250 mM NaCl, indicating that the Na^+ exclusion mechanism was effective at moderate salt stress, but did not manage to maintain non-toxic Na^+ levels at the higher salinity level. Therefore, the difference in exclusion efficiency at 250 mM NaCl in the genotypes may link to salt tolerance traits such as senescence.

4.2 The influence of the rhizome on salinity tolerance

For a perennial with a rhizome, belowground traits need to include not only the roots but also the rhizomes, which are responsible for storing nutrients including carbohydrates and proteins and survive stressful periods, such as winter time (Karp and Shield, 2008). When plants regenerate new shoots, rhizomes support nutrients for fast growth. Fonteyne et al. (2016b) studied the effect of rhizomes on frost tolerance of *Miscanthus* and did not find consistent results. It is not straightforward to compare the salt tolerance for the different genotypes with or without rhizomes, due to differences in rhizome morphology, size and composition. For example, *M. sinensis* has small and woody rhizomes, and the rhizome dry weight of *Miscanthus* × *giganteus* is typically double that of *M. sinensis* under normal field conditions (Christensen et al., 2016). In addition, the water content in rhizomes of *M. sinensis* is lower than that of *Miscanthus* × *giganteus* rhizomes (Fonteyne et al., 2016a). Plazek et al. (2014) reported that the size of the rhizome of *Miscanthus* × *giganteus* (OPM-9) affected salt tolerance. Plazek et al. (2014) used 20-30 g rhizomes and (Stavridou et al., 2016) applied approximately 20 g rhizomes of *Miscanthus* × *giganteus* for salt stress; *M. sinensis* genotypes cannot produce rhizomes as big as *Miscanthus* × *giganteus*. In order to avoid variation based on rhizome size, we used similar-sized rhizomes (5-10 g) from three-year-old plants for each genotype. Stavridou et al. (2016) reported that the rhizome dry weight of *Miscanthus* × *giganteus* was not significant different between plants grown from 0 to 240 mM NaCl but the root dry weight was significantly affected at 240 mM NaCl. A similar result was found in our study; the reference genotype *Miscanthus* × *giganteus* (OPM-9) showed a decrease in root dry weight but not in rhizome dry weight at 250 mM NaCl. We also did not observe significant differences in rhizome dry weights in most of the other genotypes at 0, 150, and 250 mM NaCl. The most salt-sensitive genotype of the rhizome-based plants, genotype OPM-42, showed a decrease in rhizome dry weight (Supplement Table 1). The trial discussed in this study was relatively short, and the responses and changes of the rhizome to salinity may be only short-term physiological responses. Under natural conditions, the rhizomes of common reeds start to produce new shoots after a period of salinity once the salinity decreases because of heavy rains or flooding (Bart and Hartman, 2003). The response of the *Miscanthus* rhizome to a long period of salinity, and its role in growth recovery and new shoot formation still needs to be explored.

Seven genotypes (OPM-7, 31, 37, 49, 56, 67, and 86) with rhizomes demonstrated higher salt tolerance than *in vitro*-started plants while two genotypes (OPM-9 and 48) with rhizomes were less salt tolerant than plants without rhizomes. Because different genotypes may use different mechanisms to deal with salt stress, and genotypic variation in rhizome-related traits may also affect salt tolerance, exploring the composition of rhizomes may contribute knowledge to rhizome-involved salt tolerance strategies, and offer opportunities for development of rhizome-started varieties for cultivation on saline lands.

Several interesting observations in our salinity tolerance evaluation may shed light on the influence of the rhizome on *Miscanthus* salt tolerance. Senescence in rhizome-based plants was less than that of *in vitro*-started plants after both 2 weeks and 6 weeks of salt treatment (Figure 1e and f), indicating that the rhizome may protect leaves of the plant from salt-induced senescence, which may improve salt tolerance. Premature senescence in leaves is known to be induced by abiotic stress factors including drought, salinity, extreme temperature and nutrient deprivation (Zimmermann and Zentgraf, 2005). The delayed senescence in the rhizome-based plants was already observed at an early stage of the stress (2 weeks of salt treatment) and already visible at the lower salt stress level (150 mM NaCl), indicating rhizomes may enhance osmotic tolerance under salt stress. Osmotic stress results in water shortage of the plant, and this can be overcome by maintenance of cellular water status by osmotic adjustment. This can be accomplished by production of organic osmolytes like proline and sugars, in addition to ion uptake, and adaptation of ion homeostasis. Common reeds produced a large number of the starch granules at the shoot base in response to salt stress (Kanai et al., 2007) and this may support compatible solutes for osmotic tolerance. It is possible that rhizomes, as storage organ, can make compatible solutes such as sugars or amino acids available to facilitate osmotic adjustment or osmotic protection. These extra compatible solutes from rhizomes may help to delay the senescence of rhizome-based plants, compared to *in vitro*-started plants.

The rhizomes did not influence the ion contents in the shoots under salt stress, but may interact with the effects of ion accumulation on growth and leaf physiology. Senescence of the *in vitro*-started plants was strongly positively correlated with leaf Na^+ (r =0.79) and Cl^- (r =0.80) and negatively correlated with K^+/Na^+ ratio (r =-0.71), while there was no significant correlation between senescence and leaf ion data in the rhizome-based plants. This remarkable result seems to indicate that rhizomes affect the sensitivity of the plants to high ion concentrations in the leaves. Nutrient deficiency, and in particular nitrogen deprivation, is one of the major factors that adversely affect plant life (Gan and Amasino, 1997). The competition of NO_3^- and Cl^- uptake is common in plants (Xu et al., 1999) so increasing nitrate nutrition is considered as a method to reduce chloride toxicity under salt stress, and increased Cl^- levels can impair nitrate uptake by the roots (Bar et al., 1997). For example, high NO_3^- supply in avocado and citrus reduced chloride accumulation and toxic symptoms in the leaves (Bar et al., 1997). Rhizomes as storage organs may compensate for the poor NO_3^- uptake, and alleviate the effect of NO_3^- deprivation resulting from high Cl^- levels. The significant relationship between senescence and leaf Cl^- may therefore only be present in the *in vitro*-started plants because rhizomes possibly can supply extra NO_3^- to delay senescence and at least partly alleviate the Cl^- toxicity.

In addition to osmotic stress and ionic stress, salinity causes oxidative stress, which can also induce senescence and increase the production of reactive oxygen species (ROS) (Das et al., 2015). Plants use different antioxidant enzymes to prevent oxidative damage in cells, which may result in delayed senescence and ultimately abiotic stress tolerance (Hasegawa et al., 2000). Some reports have shown that soluble sugars help to protect against ROS damage (Sperdouli and Moustakas, 2012). Rhizome-started plants may support mobilization of carbohydrates for detoxification of ROS in the leaves, and this may be one of the reasons why the rhizome-started plants showed less senescence than *in vitro*-started plants.

4.3 Useful traits for salt tolerance

Improving salt tolerance in crops needs to take into account both osmotic and ionic tolerance (Genc et al., 2010). For bioenergy crops, high yield but relatively low ion contents are demanded. In our tested *Miscanthus* genotypes, shoot dry weight showed a highly significant correlation with height, stem height and tiller number under saline conditions (Table 3). Because the stem is the major part of the harvest as combustion fuel, the traits linked to stem dry weight are the most important and useful indicators for breeding combustion-targeted cultivars for saline environments. In our evaluation, stem dry weight was highly positively linked to stem height and tiller number, and the best candidate genotype for producing a combustion-targeted harvest under saline conditions was genotype OPM-56. This genotype combined the highest shoot dry weight, the longest stem (101 cm vs 59 cm of the average), and the highest tiller number (6.4 vs 2.9 of the average) of the 12 genotypes with low levels of ions in its stem after 6 weeks of high salinity (250 mM NaCl).

Ion toxicity is induced by the accumulation of high ion concentrations in plant tissues, especially leaves (Munns and James, 2003). The leaf blades are more sensitive to ion toxicity than the roots so it is important for leaves to maintain a relatively low Na^+ concentration in the cytosol by adapting ion transport and homeostasis in glycophytes (Munns and Tester, 2008). Because high Na^+ concentrations interfere with K^+ uptake and K^+ function (Shabala and Cuin, 2008), maintaining a high K^+/Na^+ ratio is considered an indicator of salt tolerance under salt stress (Munns and James, 2003; Krishnamurthy et al., 2007). In our evaluation, the leaf K^+/Na^+ ratio at 250 mM NaCl was positively correlated (r =0.82) to shoot dry weight in the rhizome-derived plants, and negatively correlated (r =-0.71) to senescence in the *in vitro*-started plants. This indicates that maintaining a relatively high K^+ content compared to Na^+ contents may be an important mechanism in *Miscanthus*, but as discussed before, the origin of the plant material seems to interact with the consequences for growth and leaf physiology of the low K^+/Na^+. Genotypes with a high Na^+ content in the roots and low Na^+ contents in the leaves are likely to utilize a Na^+ exclusion mechanism that may improve salt tolerance (Møller et al., 2009). Indeed, low Na^+ content in the shoots was successfully used as selection criteria for breeding salt tolerant cultivars in wheat, barley and rice (Lin et al., 2004; Lindsay et al., 2004; Xue et al., 2009; Genc et al., 2010; Thomson et al., 2010; Munns et al., 2012). In our evaluation, genotypes OPM-42, 48, and 56 had 3 fold higher Na^+ ion concentrations in roots than in leaves at 250 mM NaCl, implying that the Na^+ exclusion mechanism was still effective after 6 weeks of high salt stress in these genotypes. Na^+ exclusion was shown to involve members of the HKT gene family. Some HKT gene family members encode Na^+ selective transporters that regulate the Na^+ concentration in plants. The HKT1;5 transporter pumps Na^+ from the xylem to parenchyma cells in the roots to prevent Na^+ accumulation in the shoots (Maser et al., 2002; Husain et al., 2003b). The leaf Na^+ concentration was highly associated with HKT1;5 allelic variation and seven major and three minor alleles of OsHKT1;5 were identified in rice across diverse accessions (Platten et al., 2013). The *PhaHKT1* gene also has been identified in reed plants (*Phragmites australis*), which are salt tolerance and perennial grasses (Takahashi et al., 2007). Similarly, a *Miscanthus* HKT1;5 ortholog may be responsible for the variation in Na^+ shoot content in *Miscanthus*. Therefore, it would be worthwhile to examine the role of *Miscanthus* HKT1;5 in Na^+ shoot exclusion in different *Miscanthus* genotypes under saline conditions.

There was a high correlation (>0.94) between Na^+ and Cl^- in roots, stems and leaves at 250 mM NaCl (Table 3). Cl^- and Na^+ uptake are often linked for electrochemical balance under stress (Teakle and

Tyerman, 2010). However, the average Cl^- root/shoot ratio was 1 but the Na^+ root/shoot ratio was 2.3 in the *in vitro*-started plants, and the average Cl^- root/shoot ratio was 1.1 but the Na^+ root/shoot ratio was 2.0 in rhizome-derived plants. This indicates that there is an active Na^+ exclusion mechanism, but the Cl^- contents are not strictly linked in *Miscanthus*. It was shown previously that mineral concentration of *Miscanthus* varied significantly depending on genotype, harvest time, location, and fertilization (Brosse et al., 2012). Cl^- and K^+ start to accumulate in dry matter even under normal fields conditions (Jorgensen, 1997), which decrease the combustion quality of *Miscanthus* (Lewandowski et al., 2003). Our results in this chapter and in chapter 2 clearly show that the accumulation of ions in *Miscanthus* biomass is significantly higher under salt stress, but also shows that there is genetic variation for ion accumulation under saline conditions, offering the possibility for improvement of this trait through breeding. The high yielding and low ion containing genotype OPM-56 under salt stress may therefore also be considered as a potential breeding material for improvement of both salt tolerance and combustion quality under saline cultivation.

4.4 Preferred genotypes

Miscanthus × *giganteus* with its high yield is the most popular commercial genotype. The average salt tolerance of *Miscanthus* × *giganteus* (OPM-9) of two types of materials was 63% at 250 mM NaCl, which was higher than the average (54%) among 12 genotypes in our experiment and might indicate that this genotype is a good choice for saline cultivation. However, genotype OPM-9 performance was quite different, almost contrasting performance for the *in vitro*- and rhizome-propagated plants: it showed 38% salt tolerance in rhizome-started plants and 88% in the *in vitro* plants. In addition, genotype OMP-9 was highly senesced in *in vitro*-plants, indicating that prolonged salinity may affect this genotype more strongly than others.

Ion contents of harvested material interfere with combustion quality because the high mineral contents not only reduce the ash melting point but also cause corrosion issues, especially K^+ and Cl^- (Brosse et al., 2012). The K^+ and Cl^- concentrations in senesced material in spring is reduced by 85-95% under normal field conditions due to relocation of nutrition and minerals to storage organs such as rhizomes, and leaching (Jorgensen, 1997). However, it is largely unknown how much minerals still remain in senesced stems grown on saline lands at harvest time. Therefore, planting genotypes with salt exclusion mechanisms under salt conditions may be a better choice; the low ion contents in shoots not only benefit for growth but also improve the combustion quality. Genotype OPM-9 (*Miscanthus* × *giganteus*) had a relatively high accumulation of Na^+ and Cl^- in the leaves, while two *M. sinensis* genotypes (OPM-48 and OPM-56) had relatively low ion contents in the shoots at 250 mM NaCl, and these are better candidates as starting materials for breeding programs aiming at improving salinity tolerance. Genotype OPM-56 also had low K^+ contents in stem and leaves, which is likely to favour product quality as well. Therefore, genotype OPM-56 stands out as a genitor for salt tolerance in *Miscanthus* breeding, as well as a favourable genotype for growth under saline conditions. It should be noted that the salinity tolerance of genotype OPM-56 and the other genotypes needs to be validated under prolonged saline conditions up until senesced harvest, and in the field.

5 Funding

The research received funding from the European Union consortium OPTIMISC (project ID 289159).

Chang-Lin Chen is supported by a scholarship from the Ministry of Education, Taiwan.

6 Acknowledgments

This work was performed as part of the OPTIMISC project, which received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement No. 289159. The authors gratefully acknowledge expert advice from Oene Dolstra, Clifton-Brown, J.C on choice of materials, and the expert assistance with the cultivation of Geurt Versteeg and Unifarm. We thanked ILVO for supplying rhizomes of different *Miscanthus* genotypes.

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Supplementary Material

Supplementary Table 1. The rhizome dry weight of 12 genotypes in rhizome-started plants at three different salt levels after 6-week treatment.

Genotype	0 mM NaCl			150 mM NaCl			250 mM NaCl		
	Mean	±SE		Mean	±SE		Mean	±SE	
OPM-7	4.60	0.54	a	4.80	0.53	a	4.57	0.32	a
OPM-9	7.08	0.66	a	6.27	0.73	a	4.80	0.35	a
OPM-31	5.59	0.34	a	5.57	0.44	a	4.67	0.28	a
OPM-37	4.34	0.53	a	3.96	0.26	a	2.86	0.26	a
OPM-42	3.83	0.19	a	2.76	0.10	b	2.50	0.25	b
OPM-48	3.22	0.35	a	3.08	0.39	a	3.15	0.29	a
OPM-49	3.05	0.14	a	3.18	0.47	a	2.93	0.28	a
OPM-56	3.94	0.53	a	3.63	0.21	a	3.11	0.32	a
OPM-67	4.23	0.46	a	3.46	0.24	a	3.20	0.39	a
OPM-71	4.69	0.93	a	3.79	0.29	a	3.20	0.44	a
OPM-76	3.83	0.30	a	3.25	0.23	a	2.90	0.31	a
OPM-86	4.21	0.65	a	3.87	0.32	a	4.29	0.24	a

Different letters indicate significant differences between salt treatments in each genotype (LSD $_{0.05}$ test)

Supplementary Table 2. Senescence of 12 genotypes in rhizome-derived plants (R) and *in vitro*-started plants (IV) after 2-week treatment at three different salt levels.

Genotype	0 mM NaCl					150 mM NaCl					250 mM NaCl				
	R		IV			R		IV			R		IV		
	Mean	±SE	Mean	±SE	Sig.	Mean	±SE	Mean	±SE	Sig.	Mean	±SE	Mean	±SE	Sig.
OPM-7	1.66	0.23	2.23	0.20		2.13	0.02	2.84	0.12	***	2.43	0.26	3.38	0.20	*
OPM-9	1.25	0.15	2.6	0.20	***	2.27	0.10	3.61	0.17	***	2.34	0.17	3.63	0.28	**
OPM-31	1.81	0.28	1.87	0.29		2.58	0.33	3.45	0.15		2.75	0.37	3.6	0.30	
OPM-37	1.8	0.37	1.63	0.22		2.38	0.21	3.27	0.09	**	2.56	0.24	3.01	0.25	
OPM-42	1.68	0.23	1.69	0.26		2.52	0.32	3.2	0.29		3.17	0.32	2.61	0.06	
OPM-48	1.04	0.04	1.5	0.17	*	1.56	0.28	2.87	0.27	*	1.96	0.19	3.18	0.25	**
OPM-49	1.37	0.30	1.78	0.27		2.59	0.19	3.58	0.20	*	2.57	0.39	3.68	0.36	
OPM-56	2.11	0.41	2.03	0.11		2.73	0.24	2.92	0.35		2.09	0.07	3.73	0.24	***
OPM-67	1.9	0.30	2.19	0.04		2.67	0.34	3.49	0.13		2.32	0.20	3.15	0.23	*
OPM-71	1.7	0.25	2.26	0.48		2.09	0.21	3.55	0.21	**	2.24	0.24	4.04	0.15	***
OPM-76	1.76	0.26	1.84	0.31		2.71	0.34	3.84	0.10	*	3.07	0.25	3.94	0.36	
OPM-86	1.98	0.33	2.21	0.23		2.29	0.02	2.79	0.08	***	2.34	0.06	3.5	0.25	*

*;**;***: significant at P<0.05; 0.01; 0.001 respectively

Supplementary Table 3. The Na$^+$ content (mg/g DW) in leaves and stems of 12 genotypes of the *in vitro*-started plants (IV) and rhizome-based plants (R) at three different salt levels.

Genotype	Leaf						Stem					
	R			IV			R			IV		
	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM
OPM-7	1.81 a	1.19 a	4.42 ab	1.45 a	1.31 a	9.82 abc	1.12 a	1.74 a	4.62 abc	2.26 ab	2.29 ab	9.86 ab
OPM -9	4.01 a	1.43 a	15.51 abc	2.64 a	2.59 a	30.14 d	2.86 ab	4.23 abc	17.31 f	5.82 ab	4.51 ab	31.78 c
OPM -31	5.33 ab	3.91 a	20.46 bc	3.79 a	4.22 a	9.27 abc	5.30 abcd	5.56 abcd	13.74 cdef	5.17 ab	2.63 ab	9.57 ab
OPM -37	3.70 a	3.79 a	15.02 abc	2.72 a	3.05 a	9.41 abc	3.49 ab	4.47 abc	12.44 bcdef	4.46 ab	3.70 ab	10.20 ab
OPM -42	4.11 a	3.71 a	13.15 abc	3.03 a	2.17 a	5.65 a	4.28 abc	4.44 abc	8.97 abcdef	3.65 ab	2.37 ab	6.53 ab
OPM -48	5.37 ab	4.91 ab	14.62 abc	3.35 a	1.97 a	2.55 a	7.01 abcde	8.60 abcdef	15.82 ef	5.35 ab	3.59 ab	6.47 ab
OPM -49	3.74 a	1.84 a	16.87 abc	3.84 a	2.68 a	18.26 bcd	3.90 abc	4.11 abc	15.17 def	5.70 ab	2.19 a	14.65 b
OPM -56	4.77 ab	3.00 a	3.58 a	2.31 a	2.11 a	4.78 a	4.50 abc	4.25 abc	6.74 abcde	2.39 ab	2.48 ab	4.94 ab
OPM -67	3.63 a	5.33 ab	10.20 abc	2.44 a	3.47 a	6.35 abc	4.03 abc	5.52 abcd	6.49 abcde	4.87 ab	2.62 ab	6.45 ab
OPM -71	3.59 a	2.65 a	25.01 c	2.61 a	3.21 a	19.25 cd	3.93 abc	4.78 abc	16.63 ef	3.42 ab	2.19 a	13.04 ab
OPM -76	4.00 a	5.76 ab	12.02 abc	2.27 a	3.58 a	8.45 abc	2.47 ab	4.03 abc	8.57 abcdef	1.94 a	1.43 a	4.77 ab
OPM -86	4.55 ab	6.01 ab	11.24 abc	2.91 a	4.26 a	12.95 abc	4.91 abc	4.67 abc	10.99 abcdef	3.06 ab	2.53 ab	8.88 ab

Different letters indicate significant differences between salt treatments and genotypes (LSD $_{0.05}$ test)

Supplementary Table 4. The Cl^- content (mg/g DW) in leaves and stems of 12 genotypes of the *in vitro*-started plants (IV) and rhizome-derived plants (R) at three different salt levels.

Genotype	Leaf						Stem					
	R			IV			R			IV		
	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM
OPM-7	1.47 a	4.00 ab	11.47 abcde	1.82 a	5.57 abc	18.72 bcde	1.16 a	5.11 ab	8.34 abcd	3.09 ab	5.27 ab	12.82 abcd
OPM -9	2.62 ab	4.48 ab	28.32 cdef	4.67 abc	10.2 abcd	47.35 f	3.32 ab	9.41 abcde	27.40 g	8.10 abc	10.50 abcd	43.69 e
OPM -31	4.83 ab	8.60 abcd	31.90 ef	4.46 abc	8.20 abcd	16.37 abcde	6.45 abc	13.37 abcde	28.40 g	7.71 abc	8.78 abc	16.08 bcd
OPM -37	2.26 ab	7.58 abc	29.75 def	2.52 ab	7.68 abcd	20.56 cde	1.85 a	7.58 abc	20.83 efg	3.19 ab	7.88 abc	13.96 abcd
OPM -42	2.68 ab	4.85 ab	20.35 abcdef	2.57 ab	4.89 abc	11.03 abcd	4.19 ab	7.44 abc	20.87 efg	3.20 ab	6.42 ab	8.58 abc
OPM -48	2.60 ab	9.57 abcd	22.18 abcdef	3.39 ab	6.56 abcd	8.47 abcd	5.82 abc	17.73 cdefg	26.67 fg	5.39 ab	8.61 abc	10.61 abcd
OPM -49	3.07 ab	5.80 ab	32.00 ef	3.95 abc	6.86 abcd	32.86 ef	4.67 ab	10.89 abcde	28.90 g	7.06 ab	9.52 abcd	23.15 d
OPM -56	2.17 ab	8.12 abcd	6.93 abc	2.50 ab	5.68 abc	9.38 abcd	1.83 a	6.04 abc	7.52 abc	0.94 a	4.24 ab	5.72 ab
OPM -67	3.42 ab	9.16 abcd	16.62 abcdef	3.14 ab	5.49 abc	10.14 abcd	4.45 ab	7.47 abc	10.45 abcde	7.60 abc	7.66 abc	11.15 abcd
OPM -71	2.55 ab	5.69 ab	40.46 f	2.99 ab	6.95 abcd	32.78 ef	3.44 ab	8.97 abcde	26.49 fg	2.77 ab	7.56 abc	21.27 cd
OPM -76	3.23 ab	12.30 abcde	23.82 bcdef	2.88 ab	6.23 abcd	17.65 abcde	1.99 a	7.24 abc	14.49 bcdef	2.49 ab	5.27 ab	9.01 abc
OPM -86	3.84 ab	10.36 abcde	20.67 abcdef	3.44 ab	8.21 abcd	22.94 de	4.62 ab	9.87 abcde	20.34 defg	4.37 ab	9.14 abcd	15.89 bcd

Different letters indicate significant differences between salt treatments and genotypes (LSD $_{0.05}$ test)

Supplementary Table 5. The K^+ content (mg/g DW) in leaves and stems of 12 genotypes of the *in vitro*-started plants (IV) and rhizome-derived plants (R) at three different salt levels.

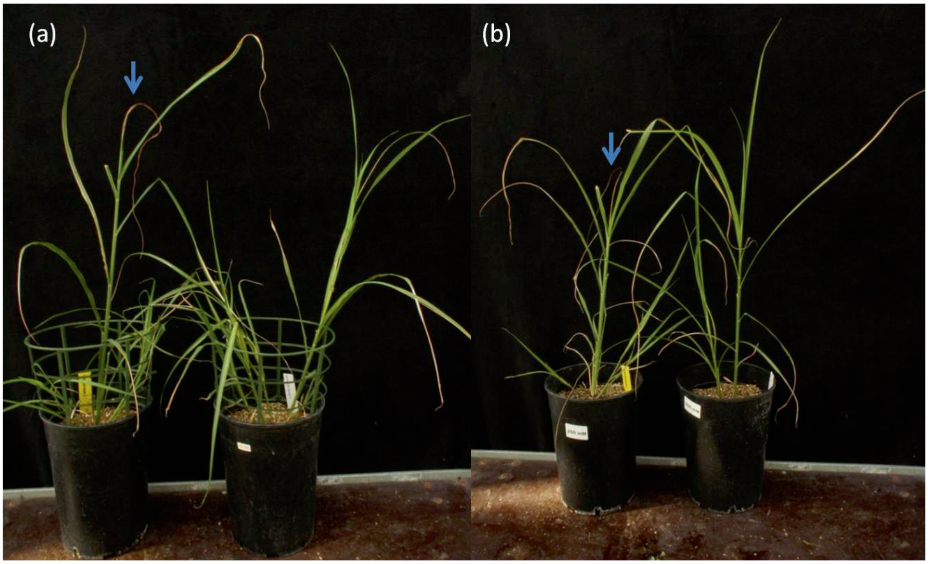
Genotype	Leaf						Stem					
	R			IV			R			IV		
	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM	0 mM	150 mM	250 mM
OPM-7	8.31 abcdefg	4.70 ab	9.93 abcdefg	7.86 abcdefg	7.36 abcde	11.61 cdefgh	7.76 ab	9.21 abc	9.89 abcde	12.26 abcde	10.29 abcd	9.65 abcd
OPM -9	11.17 abcdefg	5.37 abc	14.01 defg	12.28 defgh	9.92 abcdefgh	15.25 h	8.58 abc	10.71 abcde	15.83 bcdefg	15.18 abcde	12.88 abcde	17.76 cde
OPM -31	15.02 defg	6.78 abcd	12.19 bcdefg	13.92 fgh	7.06 abcde	9.59 abcdefgh	12.75 abcdefg	14.41 abcdefg	21.78 g	18.77 de	15.22 abcde	14.65 abcde
OPM -37	9.19 abcdefg	4.98 ab	15.29 fg	7.65 abcdef	7.09 abcde	12.39 efgh	8.83 abc	8.77 abc	15.71 bcdefg	10.97 abcd	13.24 abcde	11.98 abcde
OPM -42	7.88 abcdefg	3.00 a	9.72 abcdefg	6.54 abcde	5.28 abc	7.75 abcdef	10.97 abcdef	10.65 abcde	20.67 fg	8.58 abc	12.52 abcde	9.54 abcd
OPM -48	12.62 bcdefg	7.98 abcdefg	8.77 abcdefg	10.98 bcdefgh	7.29 abcde	9.97 abcdefgh	19.18 defg	18.03 cdefg	17.85 cdefg	16.72 bcde	15.20 abcde	11.57 abcde
OPM -49	7.94 abcdefg	4.58 ab	15.18 efg	10.14 bcdefgh	5.07 ab	14.30 gh	12.12 abcdefg	12.59 abcdefg	19.46 defg	20.86 e	15.53 abcde	14.04 abcde
OPM -56	9.60 abcdefg	6.99 abcde	4.90 ab	6.17 abcde	5.45 abc	6.72 abcde	5.70 a	7.35 ab	7.26 ab	6.04 a	7.55 ab	5.84 a
OPM -67	7.95 abcdefg	5.07 ab	8.59 abcdefg	6.52 abcde	3.62 a	6.39 abcde	10.50 abcde	8.70 abc	11.97 abcdefg	16.78 bcde	13.55 abcde	12.16 abcde
OPM -71	9.33 abcdefg	5.15 ab	16.01 g	8.79 abcdefgh	6.27 abcde	15.14 h	9.69 abcd	11.49 abcdef	19.64 efg	12.47 abcde	15.37 abcde	15.34 abcde
OPM -76	9.59 abcdefg	8.30 abcdefg	13.58 cdefg	5.88 abcd	5.08 ab	11.34 bcdefgh	6.35 ab	9.65 abcd	12.33 abcdefg	8.07 abc	12.31 abcde	11.14 abcde
OPM -86	14.35 defg	7.54 abcdef	11.73 bcdefg	9.29 abcdefgh	7.27 abcde	14.73 h	12.87 abcdefg	13.34 abcdefg	17.83 cdefg	13.37 abcde	16.97 bcde	13.99 abcde

Different letters indicate significant differences between salt treatments and genotypes ($LSD_{0.05}$ test)

Supplementary Table 6. Pearson correlations of salt tolerance of 12 genotypes between hydroponics and pot conditions.

	IVP150	IVP250	RP150	RP250	IVH150
IVP150	-				
IVP250	0.83	-			
RP150	-0.4	-0.43	-		
RP250	-0.48	-0.27	0.54	-	
IVH 150	-0.09	-0.19	0.32	0.34	-

IVH150 : *in vitro*-started plants in hydroponics at 150 mM NaCl, IVP150 : *in vitro*-started plants in pots at 150 mM NaCl, IVP250 : *in vitro*-started plants in pots at 250 mM NaCl, RP150 : rhizome-based plants in pots at 150 mM NaCl, RP250 : rhizome-based plants in pots at 250 mM NaCl.



Supplementary Figure 1. Comparison of senescence in different types of material of genotype OPM-86 under different salt stress levels for 6 weeks. (a) *In vitro*-started plants (left) and rhizome-based plants (right) at 150mM NaCl. (b) *In vitro*-started plants (left) and rhizome-based plants (right) at 250 mM NaCl. Flag leaves are indicated by arrows.

Chapter 4

The influence of salinity on *Miscanthus* biomass quality for biofuel

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Keywords: ***Miscanthus*, cell wall, cellulose, hemicellulose, ionic stress, osmotic stress**

Abstract

Miscanthus as a perennial rhizomatous C4 grass is a promising bioenergy crop for the generation of biogas by gasification and heat by combustion. To avoid competition with food crops for arable land, *Miscanthus* as a biofuel resource is required to be grown on marginal lands. These marginal lands are often low in organic matter content and high in unwanted components such as salt. Salt stress may affect the composition of the cell walls of *Miscanthus* plants, which in turn can affect the efficiency of various steps in the bioenergy production chains. Genotypes with high hemicellulose content and low lignin content in their cell walls have relatively high saccharification efficiency, and low mineral content is a good characteristic for combustion. Exploring genetic variation of cell wall composition under salt stress can help to identify high-potential genotypes as breeding materials to improve salt tolerance while also being a suitable direct resource for biofuel production when grown under saline conditions. Twelve genotypes of *Miscanthus* were first evaluated for cell wall composition and ion content under control and mild saline conditions (6 weeks of 150 mM NaCl) in pots in the greenhouse. Cell walls in the stems of plants grown under saline conditions showed an increase in hemicellulose content and a reduction in cellulose content relatively to the ones under control conditions. Na^+ accumulation in the leaves was not significantly different between control conditions and 150 mM NaCl, indicating that the plants were only mildly affected and did not experience ion toxicity stress. In a second experiment, six genotypes varying in salt tolerance were grown under more severe stress conditions (200 mM NaCl for 19 weeks). Analysis of Na^+ and Cl^- content in the shoots showed a significant increase in plants grown at this salinity level compared to control conditions. Furthermore, the hemicellulose content of the leaf cell walls was decreased, and lignin content increased under salt stress conditions with respect to control conditions. Genotypes OPM-48 and 87 contain higher hemicellulose and lower lignin content than the other genotypes under salt stress and are candidates for biogas and bioethanol production. Two genotypes (OPM-42 and 87) had relatively low Na^+ and Cl^- ion accumulation levels in the shoots, and therefore may be better candidates for combustion, avoiding the problem of corrosion.

1 Introduction

Miscanthus, a perennial rhizomatous grass with C4 photosynthesis, is considered to be one of the most promising second generation bioenergy crops due to amongst others its high photosynthetic efficiency, high yield and low input demands (Dohleman and Long, 2009; Zub and Brancourt-Hulmel, 2010; Davey et al., 2016; van der Weijde et al., 2016a). Second generation biofuel is produced from non-edible lignocellulosic biomass to avoid issues with food-security (Wyman, 2007). *Miscanthus* biomass can be used to generate heat and electricity by combustion and for production of biogas by gasification (Wagner et al., 2017). The lignocellulosic feedstock of *Miscanthus* contains three main components in the cell wall: cellulose (40 to 60%), hemicellulose (20 to 40 %) and lignin (10 to 30 %) (Brosse et al., 2012). Conversion of lignocellulosic biomass into fermentable sugars that can then be converted into bioethanol is mainly dependent on cellulose and hemicellulose (Chiaramonti et al., 2012; Rajendran et al., 2017). Cellulose is difficult to degrade because of the high degree of polymerization, while hemicelluloses are easy to digest due to low degree of polymerization (Xu et al., 2012). Although lignin is a relatively small fraction of the major cell wall components, the structure and extent of cross-linking is an important factor for the efficiency of conversion of cell wall polysaccharides into fermentable sugars (Chen and Dixon, 2007; Himmel and Picataggio, 2009; Studer et al., 2011). Van der Weijde et al. (2017) showed that variation in the *Miscanthus* cell wall composition and bioconversion efficiency were highly correlated. The cell wall composition did not only show high variation between different species and genotypes, but was also influenced by harvest time and environmental factors such as drought (van der Weijde et al., 2016b; van der Weijde et al., 2017). Ideally, bioenergy crops would be cultivated on marginal lands in order to avoid competition with food crops and to negatively impact food security (Somerville et al., 2010), it is essential to consider both quantitative and qualitative aspects of the biomass produced on these lands with frequent exposure to cold, drought, and salinity. In plants exposed to abiotic stress, cell wall remodeling changes the composition and structural rigidity, which are key factors determining the techno-economic efficiency of biofuel production (Tenhaken, 2015; van der Weijde et al., 2016a).

Soil salinity is a global issue for agricultural productivity, with almost 20% of the world's irrigated land affected by salinity (Butcher et al., 2016). It was even estimated that 50% arable land will be affected by salinity in 2050 because of poor drainage and climatic change (Bartels and Sunkar, 2005). Salinity seriously reduces plant growth and development due to a combination of osmotic stress, ionic stress, and nutritional imbalance (Ashraf and Harris, 2004; Munns and Tester, 2008). Osmotic stress (the first phase of salt stress) caused by the high concentration of ions in the rhizosphere affects growth immediately, and the response of the plants has similarities to the response to drought stress

(Munné-Bosch et al., 2001). Ion toxicity stress (the second phase of salt stress) develops over time because of ion accumulation up to toxic levels within the shoots. The high levels of Na^+ concentration in the leaves can induce early senescence (Munns and James, 2003). In addition to the adverse effects on plant growth and senescence, salinity affects major metabolic processes including sugar metabolism, photosynthesis, and cell wall biosynthesis (Hassanein, 1999; Chartzoulakis and Klapaki, 2000; Munns et al., 2006; Zagorchev et al., 2014), and may therefore affect both quantity and quality of the biomass yield of *Miscanthus* grown on saline soils.

Under salt stress, plants can utilize different mechanisms to reduce damage and deal with osmotic stress and ionic stress. To maintain cell turgor, osmotic tolerance can be achieved by adapting root water uptake properties, plant hydraulics, and by adjusting the plant osmotic potential by producing osmolytes such as proline (Khatkar and Kuhad, 2000), glycine betaine (Khan et al., 2000; Wang and Nii, 2000), sugars (Kerepesi and Galiba, 2000), and polyols (Bohnert et al., 1995; Zhifang and Loescher, 2003). The lower cell turgor can also be counteracted by stiffening of cell walls to provide structural resistance and inhibit cell extension (Moore et al., 2008; Tenhaken, 2015). Osmotic stress-tolerant plants can maintain growth under water-limiting conditions because, amongst others, cell walls can be restructured and become more flexible without suffering permanent damage (Moore et al., 2008). Van der Weijde et al. (2016b) showed that the cell wall content of stem dry matter across 50 *Miscanthus* genotypes decreased on average from 73% under control conditions to 65% under drought conditions. The average cellulose content in stem tissue decreased from 51% to 46% of the neutral detergent fibers (NDF), but hemicelluloses increased from 42% to 47% NDF. The change in hemicelluloses resulted in an increase of saccharification efficiency (van der Weijde et al., 2016b). The average lignin content remained around 6% without substantial differences between drought-treated and control plants (van der Weijde et al., 2016b). Whether similar responses will be induced under salinity stress remains to be estimated. Transcriptome studies showed that the expression of a number of cell wall-related genes and transcription factors was affected in Arabidopsis and rice under salt stress (Le Gall et al., 2015). For example, an R2R3-type MYB transcription factor of rice (OsMPS) was induced by salt stress, and this gene was shown to regulate the expression of expansin and xyloglucan endotransglucosylases/ hydrolase (XTH) genes (Schmidt et al., 2013). Both expansins and XTH are involved in cell wall remodeling under different abiotic stress conditions (Cominelli et al., 2008). Studies of the biochemical changes in cell wall components under salt stress have focused on root tissue and showed salt tolerant genotypes to have a higher abundance of pectin and lignin (Sánchez-Aguayo et al., 2004; Neves et al., 2010; An et al., 2014). An increased number of lignified tracheary elements in tomato roots under salt stress may enhance the symplastic cell-to-cell pathway for water transport, which would not only impart greater selectivity but also reduce ion uptake (Sánchez-Aguayo et al., 2004). Cellulose content is reduced dramatically under salt stress because microtubules disassemble rapidly under salt stress which cause microtubule-guided cellulose synthases (CesA) complexes to stop synthesizing (Komis et al., 2002). Recently, Companion of Cellulose Synthase 1 and 2 (CC1 and CC2) were shown to sustain microtubule organization and CesA thus aid cellulose production under salt stress, and double *cc1 cc2* mutants were hypersensitive to salt stress (Endler et al., 2015; Wang et al., 2016). Although some studies demonstrated that the cell walls of maize leaves was modified in the first phase of salt stress (osmotic stress) (Uddin et al., 2013; Uddin et al., 2014), it is still largely unknown how salt stress affects the cell wall and thus biomass quality of bioenergy crops.

In this study, the effects of salt stress on the biomass quantity and quality of different *Miscanthus* genotypes were studied. The aim was (i) to investigate the impact of salinity on biomass quality, (ii) to identify which cell wall characteristics related to direct combustion or anaerobic digestion to produce biogas are affected by salt stress, and (iii) to identify genotypes or traits that can be used for cultivation and breeding for cultivars that have superior biomass quality and quantity for biofuel production under saline cultivation conditions.

2 Materials and Methods

2.1 Plant materials

The *Miscanthus* genotypes used for evaluation in this study are listed in Table 1. These genotypes showed high variation in osmotic tolerance and shoot Na^+ content from a wide range screening at 150 mM NaCl in a hydroponic system (Table 1) and had a wide range of salt tolerance (84%-41%) at 250 mM NaCl in pots (described in Chapter 3). Each genotype was propagated from *in vitro* cultured plantlets that were further propagated on a hydroponics system.

Table 1. *Miscanthus* genotypes for the pilot and main experiments.

Genotype	Supplier	Genotype	Pilot exp.	Main exp.	Osmotic tolerance	Leaf Na^+ content
OPM-7	IBERS	Hybrid (*M. sacchariflorus* × *M. sinensis*)	*		High	Medium
OPM-9	IBERS	Hybrid (*Miscanthus* × *giganteus*)	*		Medium	Low
OPM-31	IBERS	*M. sinensis*	*		Medium	Medium
OPM-37	WUR	*M. sinensis*	*		High	Low
OPM-42	WUR	*M. sinensis*	*	*	High	Medium
OPM-48	WUR	*M. sinensis*	*	*	Medium	Medium
OPM-49	WUR	*M. sinensis*	*		Low	High
OPM-56	WUR	*M. sinensis*	*	*	Medium	Medium
OPM-67	WUR	*M. sinensis*	*		High	High
OPM-71	WUR	*M. sinensis*	*	*	High	Low
OPM-76	WUR	*M. sinensis*	*	*	High	Medium
OPM-86	WUR	*M. sinensis*	*		Medium	High
OPM-87	WUR	*M. sinensis*		*	Low	High

(IBERS) Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, UK
(WUR) Wageningen University & Research, the Netherlands

2.2 Pilot experiment

For the pilot experiment, plants were watered with 0.5X Hoagland solution as control and 0.5X Hoagland with additional 150 mM NaCl as salt stress. The plantlets were transplanted from *in vitro* culture to a hydroponic system for propagation, and then transplanted to 7L pots in the greenhouse and allowed to acclimate for 3 weeks, after which NaCl was added to pots in three daily steps of 50 mM to an end concentration of 150 mM NaCl. The experiment was set up as a Randomised Complete Block Design with four replications per genotype per treatment. The salt treatment was continued for 6 weeks until the plants were harvested. The average day/night temperature was set at 25/18˚C, and the photoperiod regime was 16 hours light and 8 hours dark. The humidity was kept at 70% and additional lighting (100 Wm^{-2}) was used when the incoming shortwave radiation was below 200 Wm^{-2} in greenhouse of Unifarm, Wageningen University & Research.

2.3 Main experiment

For the main experiment, plantlets were propagated from *in vitro* cultured plantlets that were further propagated on a hydroponics system. Plants with 4-5 leaves were transplanted from the hydroponic system to 0.5 L pots with vermiculite for acclimation to outdoor conditions under a shelter at Unifarm, Wageningen University & Research. After two weeks, the plants were transplanted to 7L pots and allowed to grow for another 3 weeks until start of the stress treatment. The plants were watered with 0.5X Hoagland (control), and for the salt stress treatment NaCl was added with a 50 mM daily increase to bring the final concentration to 200 mM NaCl. The salt treatment was maintained for 19 weeks. The experiment had a Randomize Complete Block Design with six plants per genotype per treatment; each genotype had 6 replications under control conditions as well as under saline conditions.

2.4 Assessment of growth traits

Plant height was measured from the base of the plant to the tip of the youngest fully expanded leaf with a ruler every week until plants stopped growing. At harvest, all plants from the control and salt treatment were separated into leaves and stems. Plant shoot fresh weight was measured immediately at harvest. Different plant parts were dried in a forced-air oven at 50˚C for 2 days, and the dry weight was measured.

2.5 Ion chromatography

In order to establish the ionic changes in shoots under salt stress, tillers from each genotype were sampled at three time points (6 weeks, 12 weeks, and 18 weeks), and ion contents were measured in the whole shoots of the end harvest (19 weeks). In order to obtain enough material for ion analyses, the tiller samples were pooled by randomly combining two of the six replicate samples per genotype per treatment in one of three pools. For determination of the ion contents in the 19-week old leaves, stems and NDF (neutral detergent fiber fraction, see 2.6), samples of 6 replicates per genotype were individually ground to fine powder using a hammer mill with 1 mm sieve. Dry powders were ashed at 600˚C for 12 hours. Ash samples were dissolved by shaking for 30 minutes in 1 ml 3M formic acid at 99˚C and then diluted with 9 ml MiliQ water. The samples were shaken again at 80˚C for another 30 minutes. A final 500x dilution was subsequently prepared by mixing 0.2 ml sample solution with 9.8ml MiliQ, and the Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+} content of each tiller, leaf, stem, and NDF sample was measured using Ion Chromatography (IC) system 850 Professional, Metrohm (Switzerland).

2.6 Biochemical analysis of cell walls

The biochemical analysis of cell wall composition was conducted gravimetrically. The cellulose, hemicellulose, and acid-insoluble lignin contents in cell walls can be calculated from neutral detergent fiber (NDF), acid detergent fiber, and acid detergent lignin contents (Van Soest, 1963; Van Soest, 1967). For this, half a gram of ground powder of dried leaf material was transferred to ANKOM F57 filter bags. Then, the bags were dried overnight in an oven at 103°C to get the dry matter content. Contents of neutral detergent fiber (NDF), acid detergent fiber and acid detergent lignin (ADL) contents of stem or leaf dry matter were determined by using an ANKOM 2000 Fiber Analyzer following the protocol developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY, USA). The weight fractions of detergent fiber residues were subsequently used to calculate the total dry matter of the cell wall (NDF% dm) and to obtain the fraction of cellulose [((ADF% dm-ADL% dm)/NDF% dm) × 100%], hemicellulose [((NDF% dm-ADF% dm)/NDF% dm) × 100%], and lignin ((ADL% dm/NDF% dm) × 100%) of the total cell wall content (van der Weijde et al., 2016b). All analyses were performed in duplicate and the average was calculated and correlated with other traits.

2.7 Statistical analysis of phenotypic data

The effects of salt on the growth traits, in the pilot experiment were compared by one-way ANOVA. Analysis of variance was done to examine the effect of treatment, genotype and the genotype-by-treatment interactions for the measured parameters. The cell wall composition and ion contents of different types of samples for each genotype under control and saline conditions were assessed by two-way ANOVA. Pearson correlation coefficients (r) among all the parameters were calculated. All statistical analyses were performed using the statistical software package Genstat 15th edition (VSN International Hemel Hempstead, UK).

3 Results

3.1 Pilot experiment

A pilot experiment was carried out to establish the experimental salt stress conditions that may induce changes in *Miscanthus* cell wall characteristics. The twelve genotypes grown for six weeks saline conditions (150 mM NaCl) were affected by the salt stress treatment when compared to control conditions (0 mM NaCl) (Table 2). The average shoot dry weight of the *Miscanthus* genotypes was reduced from 15.05 g at 0 mM NaCl to 13.25 g at 150 mM NaCl. The Cl^- content in the roots was increased significantly ($P<0.001$) from 1.19 mg/g DW under control conditions to 11.65 mg/g DW at 150 mM NaCl. The leaf Cl^- contents were increased as well, but less than in roots: from 3.19 mg/g DW under control conditions to 6.87 mg/g DW under salt stress. The Ca^{2+} contents in leaves and roots were not significantly different between control conditions and 150 mM NaCl. Remarkably, there was also no significant difference in leaf Na^+ content and the K^+/Na^+ ratio between control conditions and 150 mM NaCl, while Na^+ content of the roots was increased almost 5-fold ($P <0.001$)

under 150 mM NaCl salt stress. This indicates that the *Miscanthus* genotypes successfully employed a Na^+ exclusion mechanism to avoid Na^+ accumulation in shoots at this level of salinity (see Chapter 3). The reduction in biomass, which was accompanied by increased senescence, may thus be due to osmotic stress rather than ionic stress at this salt level.

Table 2. Measured traits of 12 *Miscanthus* genotypes grown at 0 mM NaCl and 150 mM NaCl.

Trait	0 mM NaCl	150 mM NaCl	Significant
Leaf dry weight (g)	7.46	6.31	**
Stem dry weight (g)	7.68	6.94	
Shoot dry weight (g)	15.05	13.25	
Senescence (Score)	2.83	5.15	***
Root Na^+ (mg/g DW)	3.29	16.16	***
Root K^+ (mg/g DW)	9.78	7.03	***
Root Ca^{2+} (mg/g DW)	5.00	4.66	
Root Cl^- (mg/g DW)	1.19	11.65	***
Root K^+/Na^+ ratio (mg/mg DW)	3.63	0.44	***
Leaf Na^+ (mg/g DW)	2.78	2.89	
Leaf Ca^{2+} (mg/g DW)	5.23	4.13	
Leaf K^+ (mg/g DW)	8.83	6.4	***
Leaf Cl^- (mg/g DW)	3.19	6.87	***
Leaf K^+/Na^+ ratio	3.3	3.5	
Stem cellulose (% NDF)	51.63	49.03	***
Stem hemicelluloses (% NDF)	42.94	45.73	**
Stem lignin (% NDF)	5.43	5.24	
Stem cell wall content (% dm)	70.75	70.60	
Leaf cellulose (% NDF)	44.87	44.47	
Leaf hemicelluloses (% NDF)	50.00	50.42	
Leaf lignin (% NDF)	5.11	5.11	
Leaf cell wall content (% dm)	64.78	65.67	

DW: Dry Weight, dm: dry matter, and NDF: Neutral Detergent Fiber; *, **, ***, significant at $P < 0.05$; 0.01; 0.001, respectively.

Cell wall characteristics were slightly affected by the 150 mM NaCl treatment. The salt-treated plants decreased cellulose content of the cell wall in stems and increased the hemicellulose content in stems compared to control plants. There was no significant change in cellulose content and hemicellulose content in leaves between 0 and 150 mM NaCl. These small changes in cell wall biomass composition showed the same tendency as the changes in *Miscanthus* cell walls induced by drought stress (van der Weijde et al., 2016b), and indicate that salinity may affect cell wall composition.

To gain further insight in the impact of both components of salt stress (osmotic stress as well as ionic stress) on quality for biofuel production, another experiment was performed in which the salt severity was increased from 150 mM NaCl to 200 mM NaCl to induce ionic stress, and six genotypes were selected with different salt tolerance responses (Genotype OPM-48, 56, and 71 showed high salt tolerance and OPM-42, 76, and 87 had medium salt tolerance). Additionally, the treatment period was extended from 6 weeks to 19 weeks to allow further accumulation of ions, and to enable harvesting of the biomass at an economically more relevant plant stage (senesced plants).

3.2 Effects of salinity on growth and yield

The 19-week 200 mM NaCl salt stress treatment caused a much stronger reduction in shoot dry weight and height than the 6 weeks of 150 mM NaCl treatment used in the pilot experiment. The average shoot dry weight decreased from 19.97 g under control conditions to 4.72 g under salt stress (Figure 1A). The stem/leaf ratio significantly decreased from 0.26 under normal conditions to 0.14 at 200 mM NaCl (Figure 1B). Average plant height significantly decreased from 119.6 cm under normal conditions to 84.2 cm under salt stress (Figure 1C).

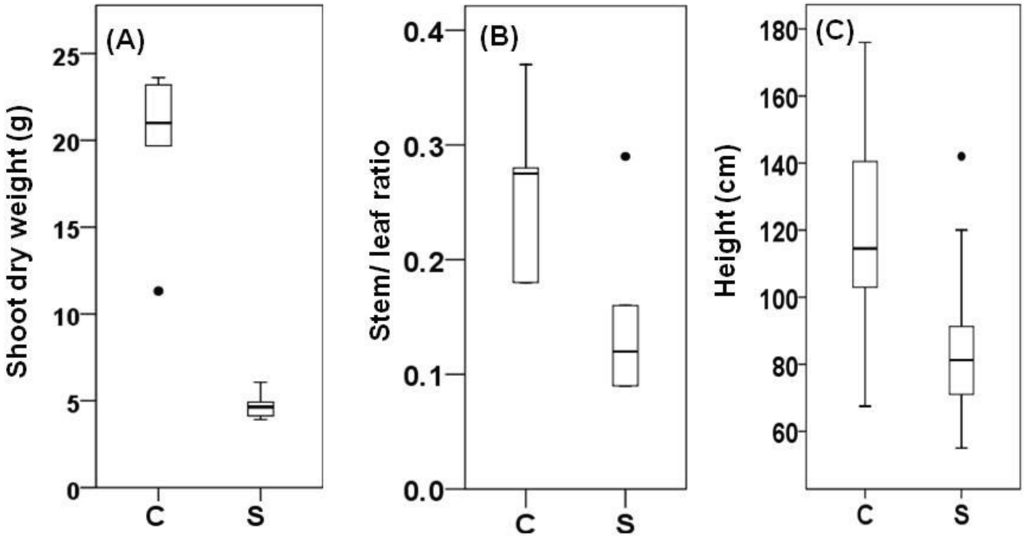

Figure 1. Box plots of (A) shoot dry weight, (B) stem DW/leaf DW ratio and (C) plant height under control conditions (C) and salt stress (S). Box edges show upper and lower quartile and the median is shown in the middle of the box. Outliers are shown as dots.

Salt tolerance was calculated as the percentage of shoot dry biomass production under saline conditions relative to control conditions. The genotypes ranged in salt tolerance from 20% in OPM-56 to 37% in OPM-76 (Table 3). Although genotype OPM-76 demonstrated the highest salt tolerance among the 6 genotypes, this genotype was relatively low-yielding under both control and salt treatments (Table 3). The average salt tolerance of six genotypes was 24.7% at 200 mM NaCl. Genotype OPM-56 had relatively low shoot biomass with 19.68 g under control conditions and 3.92 g under salt stress while genotype OPM-71 had relatively high biomass with 23.2 g at 0 mM NaCl and 6.07 g at 200 mM NaCl. Genotype OPM-42 has the highest stem dry weight at 200 mM NaCl and also relatively high stem dry weight under control conditions. Genotype OPM-71 showed the second highest stem dry weight under salt stress, while the other 4 genotypes had no comparable stem dry weights at 200 mM NaCl (Table 3). For leaf dry weight at 200 mM NaCl, genotype OPM-71 produced the most biomass (5.31 g), while genotype OPM-56 had relatively low biomass (3.39 g). Five genotypes showed a significant decrease in stem/leaf ratio, but for genotype OPM-42 there was no significant change (Table 3). Genotype OPM-42 had the highest stem/leaf ratio (0.29) under salt stress, while genotype OPM-48, 76 and 87 had the lowest stem/leaf ratio under salt stress.

Table 3. The leaf dry weight, stem dry weight, stem/leaf ratio, shoot dry weight and salt tolerance of 6 *Miscanthus* genotypes under control and salt stress.

Gynotype	Leaf dry weight (g)		Stem dry weight (g)		Stem/leaf ratio		Shoot dry weight (g)		Salt tolerance (%)
	0 mM	200 mM	0 mM	200 mM	0 mM	200 mM	0 mM	200 mM	
OPM-42	18.34 b	3.79 a	5.27 b	1.13 c	0.27 b	0.29 b	23.61 b	4.92 ab	21
OPM-48	17.46 b	4.33 ab	3.21 ab	0.37 a	0.18 a	0.09 a	20.67 b	4.70 a	23
OPM-56	14.34 ab	3.39 a	5.34 b	0.53 ab	0.37 c	0.16 a	19.68 b	3.92 a	20
OPM-71	18.06 b	5.31 b	5.14 b	0.76 b	0.28 b	0.14 a	23.20 b	6.07 b	26
OPM-76	9.58 a	3.76 a	1.75 a	0.38 a	0.18 a	0.10 a	11.32 a	4.13 a	37
OPM-87	16.78 b	4.09 a	4.55 b	0.39 a	0.28 bc	0.09 a	21.33 b	4.48 a	21

The means (n =6) marked with the same letters in the same column do not have significant differences between genotypes (multiple range Fisher's test, $p < 0.05$)

Salinity inhibited plant elongation and shortened the time to maximum height for all genotypes, but there was significant variation among the six genotypes in both control conditions and salt stress. Under control conditions, the longest time period to reach maximum plant height was 12 weeks for genotype OMP-42, and the shortest was 7 weeks for genotype OPM-76 (Figure 2A). The time to maximum plant height of genotype OPM-56 (9 weeks) was shorter than genotype OPM-48, 71 and 87 with 10 weeks (Figure 2A). The elongation period to maximum plant height of three genotypes (OPM-42, -56 and 76) was reduced under salt stress compared with control conditions (Figure 2B).

After 12 weeks of growth, genotype OPM-42 was the highest in control (158 cm) as well as salt conditions (115cm) while genotype OPM-76 was the smallest (92 cm and 65 cm in control and salt conditions, respectively) (Figure 2A and B). When comparing the height difference between control conditions and salt stress, genotype OPM-56 was affected the most and genotype OPM-48 was affected the least.

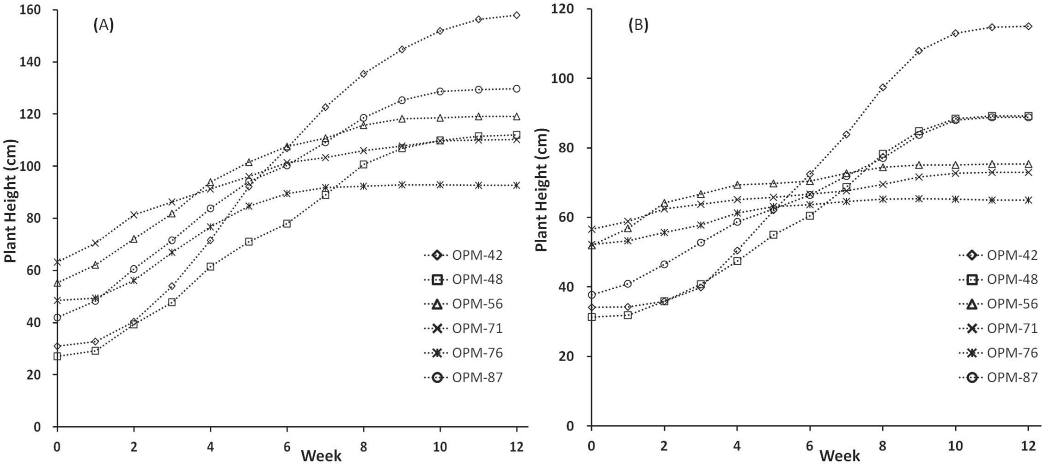

Figure 2. Changes in height (cm) over time (weeks) of 6 genotypes under control conditions (A) and 200 mM NaCl (B).

3.3 Ion homeostasis

To monitor the development of ionic stress under salt stress, ion concentrations in tillers from each genotype at three time points (6 weeks, 12 weeks, and 18 weeks) were measured and compared, and at week 19, ion contents of the whole stems and leaves separately were also measured. The Na^+ and Cl^- accumulation increased with time under salt stress (Figure 3A and B).

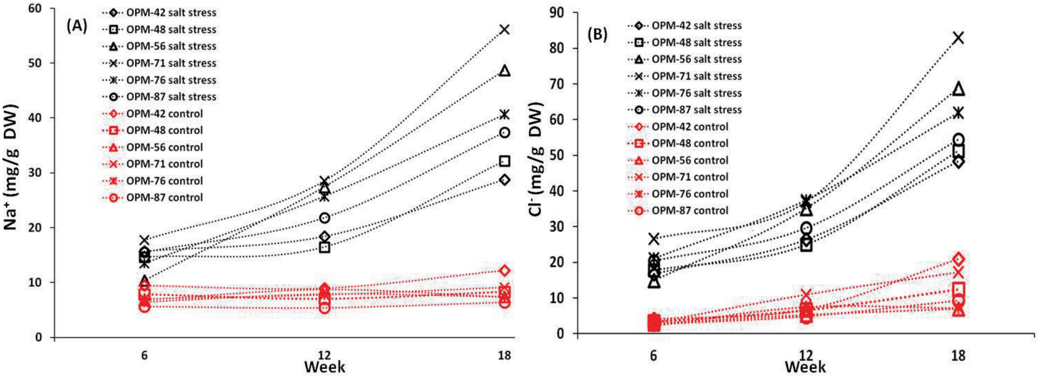

Figure 3. Tiller Na^+ (A) and Cl^- (B) contents in 6 genotypes at 0 and 200 mM NaCl over the growing period.

After 19-weeks salt treatment, the Na^+ contents in the leaves increased from 7.07 to 21.67 mg/g DW and the Cl^- contents in leaves increased from 6.79 to 31.24 mg/g DW, demonstrating that at 200 mM NaCl salinity (Figure 4A), ions accumulated in the shoot up to a level that may cause ionic stress for plants above 3-10 mg/g DW Na^+ (Ayers and Westcot, 1985), and 4-7 mg/g DW Cl^- for Cl^- sensitive species and 15-50 mg/g DW for Cl^- tolerant species (White and Broadley, 2001). The leaf K^+ content did not show significant differences between control conditions and 200 mM NaCl, while the K^+ /Na^+ ratio in the leaves decreased significantly from 0.75 under control conditions to 0.25 under salt stress (Figure 4B). After 19-weeks salt treatment, stem Na^+ contents also increased from 4.39 to 17.84 mg/g DW, and Cl^- contents increased from 1.23 to 23.05 mg/g DW, and in this tissue the K^+ contents increased significantly from 3.19 mg/g DW at 0 mM NaCl to 6.53 mg/g DW at 200 mM NaCl (Figure 4C) resulting in a K^+/Na^+ ratio that was still decreased, but to a lesser extent than in leaves, from 0.79 under control conditions to 0.42 under salt stress (Figure 4D). On average, the Na^+ and Cl^- concentrations in the leaves were higher than that in the stems under salt stress and the K^+ /Na^+ ratio in the leaves was lower than that in the stems under salt stress. Ca^{2+} contents in both leaf and stem tissues were not significantly different between salt treatments and control conditions. The Mg^{2+} contents increased from 8.41 mg/g DW at 0 mM NaCl to 10.44 mg/g DW at 200 mM NaCl for leaf tissues and increased from 2.72 mg/g DW at 0 mM NaCl to 4.75 mg/g DW at 200 mM for stems after 19-week treatment.

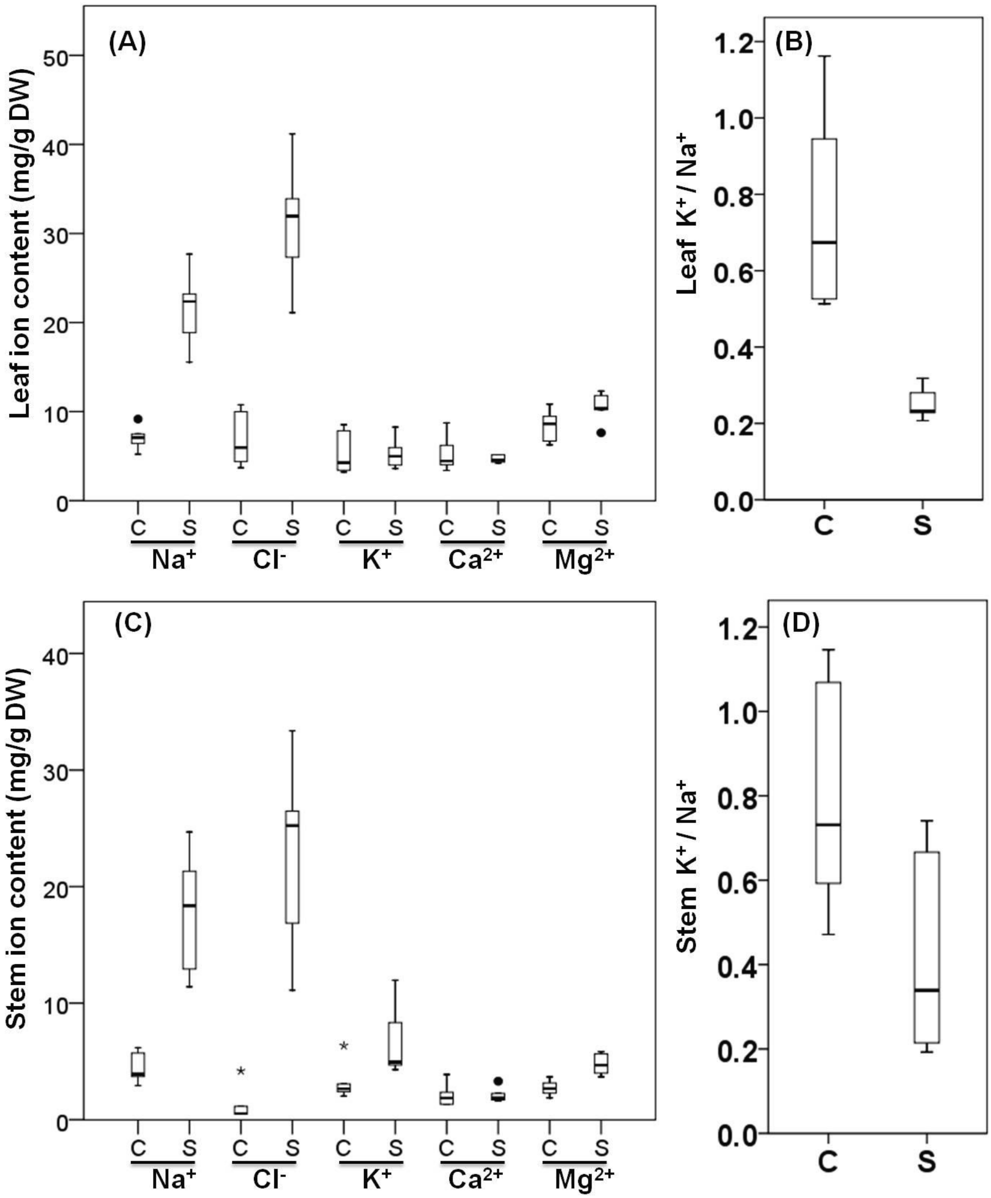

Figure 4. Analyses of ion contents (A, C) and K^+/Na^+ ratio (B, D) in leaves (A ,B) and stem (C, D) of 6 *Miscanthus* genotypes grown under salt stress (S) compared to control conditions (C) for 19 weeks. Box edges show upper and lower quartile and the median is shown in the middle of the box. Outliers are shown as dots and extreme outliers are shown as stars.

The 6 genotypes showed large differences in leaf Na^+ content of 19 weeks salt-stressed plants, from 15.55 mg/g DW in genotype OPM-42 to 27.68 mg/g DW in genotype OPM-71. Leaf Cl^- contents even ranged from 21.11 mg/g DW in OPM-42 to 41.19 mg/g DW in OPM-71 (Table 4). In the stem tissues under salt stress, genotype OPM-56 had high Na^+ and Cl^- content (24.68 mg/g DW and 33.37 mg/g DW, respectively), while genotype OPM-87 had relatively low Na^+ and Cl^- contents (11.41 mg/g DW and 11.11 mg/g DW, respectively) (Table 5). It is important to note that Na^+ and Cl^- accumulation in the genotypes at 6 weeks was not predictive for ion contents at later time points. Genotype OPM-56 had the lowest leaf Na^+ contents (10.37 mg/g DW) after 6 weeks of salt treatment but had the second highest concentration (48.7 mg/g DW) at 18 weeks under salt conditions, while genotype OPM-42 had the second highest leaf Na^+ content (15.66 mg/g DW) after 6 weeks of salt treatment but the lowest concentration (28.8 mg/g DW) at 18 weeks (Figure 3A). Cl^- accumulation showed a similar trend (Figure 3B).

Table 4. Ion contents in the leaves of 6 *Miscanthus* genotypes under control and salt stress for 19 weeks.

Genotype	Cl^- (mg/g DW)		Na^+ (mg/g DW)		K^+ (mg/g DW)		K^+/Na^+ (mg/g DW)	
	0mM	200mM	0mM	200mM	0mM	200mM	0 mM	200 mM
OPM-42	7.23 ab	21.11 c	7.30 a	15.55 b	3.73 a	3.61 a	0.51 ab	0.23 a
OPM-48	9.98 ab	33.90 d	7.48 a	22.55 cd	8.52 a	5.95 a	1.16 c	0.28 a
OPM-56	4.67 ab	30.75 d	6.85 a	22.19 cd	4.79 a	4.73 a	0.72 abc	0.23 a
OPM-71	10.77 b	41.19 e	9.16 a	27.68 e	7.85 a	8.26 a	0.95 bc	0.32 a
OPM-76	3.69 a	33.15 d	6.42 a	23.21 d	3.41 a	5.24 a	0.53 ab	0.24 a
OPM-87	4.38 ab	27.33 cd	5.21 a	18.86 bc	3.21 a	3.98 a	0.63 abc	0.21 a

The means (n = 6) marked with the same letters do not differ significantly between genotypes and treatment (multiple range Fisher's test, $p < 0.05$).

The stem K^+ contents ranged from 4.3 mg/g DW in OPM-76 to 11.97 mg/g DW in OPM-48 after 19-week salt treatment (Table 5). For producing biogas, high ion concentrations in the NDF have negative impacts for fermentation, so keeping low ion contents in the cell wall fractions is important for the biogas production process (Uygur and Kargı, 2004; Lefebvre et al., 2007). Therefore, the Na^+ and Cl^- ion contents of leaf NDF were also measured. Na^+ content showed no significant difference between control (0.98 mg/g DW) and salt stress conditions (1.14 mg/g DW), and Cl^- could not even be detected.

Table 5. Ion contents in the stems of 6 *Miscanthus* genotypes under control and salt stress for 19 weeks.

Genotype	Cl^- (mg/g DW)		Na^+ (mg/g DW)		K^+ (mg/g DW)		K^+/Na^+ (mg/g DW)	
	0 mM	200 mM	0 mM	200 mM	0 mM	200 mM	0 mM	200 mM
OPM-42	1.15 a	16.86 b	6.18 b	12.93 c	2.39 a	8.33 bc	0.47 ab	0.67 abc
OPM-48	4.19 a	26.47 c	5.72 ab	16.54 d	6.36 ab	11.97 c	1.15 c	0.74 abc
OPM-56	0.50 a	33.37 d	3.83 ab	24.68 f	2.03 a	4.69 ab	0.59 abc	0.19 a
OPM-71	0.49 a	26.32 c	4.00 ab	21.32 e	2.60 a	5.19 ab	0.66 abc	0.25 ab
OPM-76	0.49 a	24.14 c	2.92 a	20.18 e	3.07 a	4.30 ab	1.07 c	0.21 a
OPM-87	0.56 a	11.11 b	3.70 ab	11.41 c	2.68 a	4.71 ab	0.80 bc	0.43 ab

The means (n =6) marked with the same letters do not differ significantly between genotypes and treatment (multiple range Fisher's test, $p < 0.05$).

3.4 Effect of salt stress on cell wall composition

Due to the strong reduction of shoot dry weight at 200 mM NaCl, it was only possible to analyze leaves for cell wall content and composition. Changes in cell wall composition of the leaves will however likely imply that the cell wall composition in stems were also influenced by salt stress, as in the pilot experiments, stem cell wall composition was more affected than that of leaves. Averaged over the tested genotypes, the salt treatment had low but significant impact on total cell wall content (% dm), hemicellulose and lignin fractions but not on the cellulose fraction of the cell wall. On average, the cell wall content slightly decreased from 64.57% dm under control conditions to 63.13% dm under salt stress (Figure 5A). Hemicellulose content reduced from 44.62% NDF at 0 mM NaCl to 43.66% NDF at 200 mM NaCl (Figure 5C). Lignin contents increased from 6.89% NDF under control conditions to 7.37% NDF at 200 mM NaCl (Figure 5D).

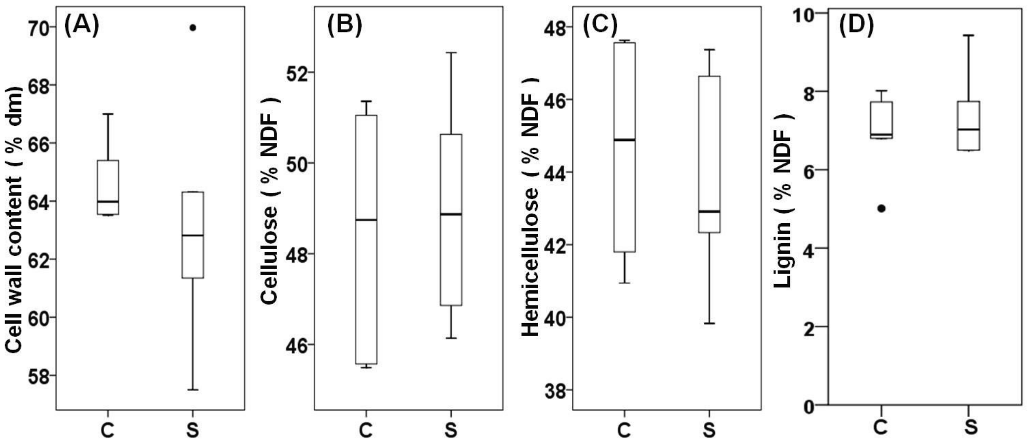

Figure 5. Box plots of leaf compositions under control (C) and salt stress (S). Cell wall content (A), Cellulose (B), Hemicellulose (C), Lignin (D). Box edges show upper and lower quartile and the median is shown in the middle of the box. Outliers are shown as dots.

The cell wall content and composition were differentially affected by salt stress in the genotypes, and genotypic variation for cell wall contents was higher in salt-treated plants compared to control plants. The cell wall content ranged from 63.51% dm in genotype OPM-76 to 67% dm in genotype OPM-42 under control conditions, but it ranged from 57.5% dm in genotype OPM-71 to 69.97% dm in genotype OPM-42 under salt stress (Table 6). The cell wall content in genotype OPM-42 slightly increased under salt stress compared with control conditions while genotype OPM-56 and OPM-71 showed a significant decrease under salt stress. Cellulose contents ranged from 45.49% NDF for genotype OPM-48 to 51.36% NDF for genotype OPM-71 under control conditions and from 46.14% NDF for genotype OPM-87 to 52.43% NDF for genotype OPM-71 under salt stress; the hemicellulose contents ranged from 40.94% NDF for genotype OPM-56 to 47.63% NDF for genotype OPM-87 under control conditions and from 39.83% NDF for genotype OPM-71 to 47.37% NDF for genotype OPM-87 under salt stress. Lignin contents ranged from 5.01% NDF for genotype OPM-42 to 8.02% NDF for genotype OPM-56 under control conditions and from 6.49% NDF for genotype OPM-87 to 9.43% NDF for genotype OPM-56 under salt stress. Genotype OPM-56 had significantly decreased cellulose contents (from 51.05% NDF to 48.24% NDF), while genotype OPM-42 increased cellulose contents from 48.52% NDF under control conditions to 50.63% NDF under salt stress (Table 6). The hemicellulose contents in genotype OPM-42 and OPM-71 were significantly reduced by the salt treatment, from 46.47% NDF to 42.76% NDF OPM-42 and from 41.8% NDF to 39.83% NDF for OPM-71, but the other four genotypes did not show significant differences between control and salt stress. Lignin contents of genotype OPM-42 and OPM-56 increased significantly under salt stress

(Table 6). Genotype OPM-48 and OPM-87 demonstrated relatively low cellulose contents and high hemicelluloses contents under control as well as salt stress. In contrast, genotype OPM-42 and OPM-71 had relatively higher cellulose contents and lower hemicellulose contents. Genotype OPM-56 had the highest lignin contents under salt stress (Table 6).

Table 6. A comparison of cell wall composition and cell wall content in leaves of six genotypes grown under 0 mM and 200 mM NaCl treatments for 19 weeks.

Genotype	Cellulose (% NDF)		Hemicellulose (% NDF)		Lignin (% NDF)		Cell wall content (% dm)	
	0 mM	200 mM	0 mM	200 mM	0 mM	200 mM	0 mM	200 mM
OPM-42	48.52 c	50.63 de	46.47 d	42.76 c	5.01 a	6.61 bc	67.00 e	69.97 f
OPM-48	45.49 a	46.86 ab	47.56 d	46.64 d	6.95 bcd	6.50 b	63.54 bcd	62.78 bc
OPM-56	51.05 ef	48.24 bc	40.94 ab	42.33 bc	8.02 d	9.43 e	65.40 de	61.35 b
OPM-71	51.36 ef	52.43 f	41.80 bc	39.83 a	6.84 bcd	7.74 cd	63.92 cd	57.50 a
OPM-76	48.97 c	49.50 cd	43.30 c	43.06 c	7.73 cd	7.45 bcd	63.51 bcd	62.85 bc
OPM-87	45.57 a	46.14 a	47.63 d	47.37 d	6.80 bc	6.49 b	64.04 cd	64.31 cd
Average	48.49	48.97	44.62	43.67	6.89	7.37	64.57	63.13

The means (n =6) of each parameter that are marked with the same letter do not differ significantly between genotypes and treatment according to multiple range Fisher's test (p <0.05).

3.5 Correlation Analysis

Pearson correlations between the measured traits and ion contents are given in Table 7. Cl^- and Na^+ contents in leaves and stems were strongly correlated at 200 mM NaCl (r =0.98 and 0.93, respectively). Both leaf Na^+ and Cl^- had negative correlations with plant height (r =-0.59 and -0.56, respectively) and stem dry weight (r =-0.37and -0.36, respectively) under salt stress. There was a high correlation between Cl^- and K^+ contents in leaves (r =0.73) and stems (r =0.81) at 0 mM NaCl but a weaker correlation in leaves (r =0.46) and no significant correlation in stems under salt stress. Leaf K^+ also had a positive correlation with leaf dry weight (r =0.44) and shoot dry weight (r =0.36). Remarkably, cell wall content was significantly and strongly negatively correlated with leaf Na^+ and Cl^- contents under salt stress conditions (both r =-0.79), while under control conditions there was no correlation with these traits. The correlation between cell wall content and plant height was stronger (r =0.79) under salt stress than that under control conditions (r =0.43). Stem K^+/Na^+ ratio had a weak but significant positive correlation of r =0.45 with cell wall content under salt stress. Cellulose content was, as expected, highly negative correlated with hemicelluloses under both control (r =-0.90) and salt stress (r =-0.88), and the hemicellulose contents were negatively correlated with lignin contents both under control (r =-0.57) and salt stress (r =-0.56). The relationship between lignin content and stem Cl^- content was not significant under control conditions but the lignin content in the leaves demonstrated a positive correlation with stem Cl^- (r =0.46) and stem Na^+ (r =0.52) under salt stress. The relationship between hemicellulose contents and stem Na^+ contents was not significant under control conditions but was significantly negative under salt stress (r =-0.49). There was a significant relationship between cellulose content and stem dry weight (r =0.45) at 200 mM NaCl but it was not significant under control conditions.

Table 7. Person's correlation coefficients between the traits of control plants (right upper triangle) and salt-treated plants (left lower triangle).

	Height	LDW	StDW	SDW	Cel	Hem	Lig	CWC	leaf Cl^-	leaf Na^+	leaf K^+	leaf K^+/Na^+	Stem Cl^-	Stem Na^+	Stem K^+	Stem K^+/Na^+
Height	-	0.34	0.32	0.35	-0.09	0.23	-0.35	0.43	0.00	-0.03	-0.19	-0.15	-0.07	0.38	-0.20	-0.34
LDW	0.17	-	0.75	0.98	-0.17	0.25	-0.26	0.48	0.31	0.04	0.04	0.05	0.12	0.10	0.04	-0.07
StDW	0.70	0.39	-	0.87	0.15	-0.08	-0.09	0.59	0.06	0.04	-0.22	-0.21	-0.12	-0.03	-0.23	-0.23
SDW	0.35	0.96	0.63	-	-0.08	0.16	-0.22	0.54	0.25	0.04	-0.04	-0.03	0.05	0.06	-0.04	-0.12
Cel	-0.03	0.13	0.45	0.25	-	-0.90	0.17	0.08	0.03	0.40	0.03	-0.09	-0.52	-0.13	-0.52	-0.41
Hem	0.18	-0.05	-0.39	-0.16	-0.88	-	-0.57	-0.05	0.11	-0.24	-0.05	0.02	0.46	0.28	0.40	0.23
Lig	-0.33	-0.12	0.03	-0.10	0.10	-0.56	-	-0.05	-0.31	-0.22	0.06	0.12	-0.06	-0.40	0.06	0.26
CWC	0.79	-0.09	0.54	0.09	-0.13	0.26	-0.32	-	-0.14	-0.06	-0.30	-0.30	-0.11	-0.03	-0.13	-0.10
leaf Cl^-	-0.56	0.15	-0.36	0.02	0.07	-0.14	0.18	-0.79	-	0.66	0.73	0.54	0.42	0.30	0.36	0.05
leaf Na^+	-0.59	0.07	-0.37	-0.05	0.10	-0.19	0.22	-0.79	0.98	-	0.23	-0.06	0.09	0.08	0.05	-0.08
leaf K^+	-0.28	0.44	-0.04	0.36	0.13	-0.20	0.18	-0.42	0.46	0.32	-	0.94	0.35	0.21	0.36	0.13
leaf K^+/Na^+	-0.02	0.41	0.09	0.37	0.04	-0.07	0.09	-0.08	0.03	-0.13	0.87	-	0.33	0.20	0.35	0.14
Stem Cl^-	-0.44	-0.22	-0.22	-0.25	0.17	-0.36	0.46	-0.50	0.58	0.61	0.23	0.00	-	0.43	0.81	0.41
Stem Na^+	-0.57	-0.17	-0.19	-0.20	0.29	-0.49	0.52	-0.59	0.59	0.65	0.22	-0.05	0.93	-	0.05	-0.45
Stem K^+	0.27	-0.08	-0.05	-0.08	-0.17	0.23	-0.20	0.14	0.03	-0.03	0.08	0.15	0.29	-0.06	-	0.82
Stem K^+/Na^+	0.56	-0.01	0.12	0.03	-0.25	0.39	-0.39	0.45	-0.28	-0.35	-0.08	0.10	-0.20	-0.53	0.85	-

SDW, Shoot dry weight; LDW, Leaf dry weight; StDW, Stem dry weight; CWC, Cell wall content (% dm); Cel, Cellulose (% NDF); Hemicellulose (% NDF); Lin, Lignin (% NDF). From light red to dark red, increasingly more positive correlation. From light blue to dark blue, increasingly more negative correlation.

4 Discussion

Miscanthus has been considered as a potential bioenergy crop in Europe already since the 1980s (Brosse et al., 2012; Chung and Kim, 2012). Its success as a biofuel crop partly depends on being able to support a stable and high-quality biomass production from marginal soils like saline soils, to avoid competition with agricultural lands used for food and feed production. Saline soils affect plant growth, and salinity levels of 120-150 mM NaCl were shown to cause 50% reduction in *Miscanthus* × *giganteus* biomass (Plazek et al., 2014; Stavridou et al., 2016). We identified in a previous study several salt tolerant *M. sinensis* genotypes in a large scale screen with 70 genotypes on a hydroponic system, showing the potential of *Miscanthus* for biomass production on saline soils (Chen et al., 2017). However, it still remained to be explored in how far cultivation on saline soils would affect the quality of the biomass. We show here that cell wall components that are important for biofuel production are affected by salinity. At a salinity level of 200 mM NaCl, *Miscanthus* genotypes accumulated Na^+ ions in the shoots and their cell wall compositions were different compared with plants grown under control conditions. The genotypic variation of ion content and cell wall composition also indicated that genotypes can be selected for biomass quality that would be less affected, and thus be better for cultivation on saline soils. Genotype OPM-87 demonstrated relatively high hemicellulose contents, low lignin contents and low ion contents in the shoots at 200 mM NaCl. This would be beneficial for the quality of biomass under salt stress for anaerobic digestion because hemicelluloses are positive factors while lignin has negative effects on biomass digestibility (Xu et al., 2012). For improvement of the combustion quality, low ion content in the biomass can prevent stoves from corroding, slagging and fouling (Baxter et al., 2012; Brosse et al., 2012). Stem dry weight is the major part that is harvested for combustion purposes after winter so the high stem/leaf ratio is considered as an important trait for combustion. Therefore, genotype OPM-42 with low ion contents, high stem dry weight and high stem/leaf ratio at 200 mM NaCl may be preferred for producing biomass for combustion under salt lands. This small set of *Miscanthus* genotypes already showed a wide range of variation for the cell wall composition under salt stress, indicating that that evaluating these traits in a more elaborate *Miscanthus* germplasm pool can accelerate breeding for various utilization purposes (Chen and Dixon, 2007; Himmel and Picataggio, 2009; Studer et al., 2011).

4.1 Plant response to salt stress

The twelve *Miscanthus* genotypes tested in the pilot experiment were able to avoid Na^+ accumulation in the shoot at 150 mM NaCl but the salinity in the root environment still resulted in growth reduction, most likely caused by osmotic stress. Plants need to make osmotic adjustments by producing compounds such as glycine betaine, soluble sugars and polyols to maintain turgor under osmotic stress (Kerepesi and Galiba, 2000; Wang and Nii, 2000; Guenni et al., 2002). This can cause a trade-off for carbon, reducing growth and cell wall synthesis (Iraki et al., 1989b; Moore et al., 2008; Emerson et al., 2014). The cell walls of tobacco cells subjected to osmotic stress were reduced in size and thickness compared to those of non-stressed cells (Iraki et al., 1989a). The slow growth and small size of cells as a response to drought could also be part of a survival strategy to maintain turgor (Bressan et al., 1982; Binzel et al., 1985). When water becomes available again, the cell walls need to restructure and expand. Therefore, a loose or plastic cell wall structure containing more hemicellulose is beneficial under a scenario of intermittent drought because hemicellulose cross-links are more easily hydrolyzed compared with lignin and cellulose (Le Gall et al., 2015; Tenhaken, 2015). A significant increase in hemicellulose content in drought-treated plants compared with control plants was observed for a diverse set of 50 *Miscanthus* genotypes (van der Weijde et al., 2016b). Similarly, a significant effect on cell wall hemicellulose content but not on cellulose and lignin was reported in *Cornus stolonifer* shoot tissue under mild salt stress (25 and 50 mM NaCl) (Mustard and Renault, 2004). Our results showed similar changes of cell wall composition in the pilot experiment. The ion data indicated that plants grown at 150 mM NaCl salinity did not suffer from ionic stress, because leaf Na^+ contents and K^+/Na^+ ratio were hardly affected (Table 2.). Glycophytes use a shoot exclusion mechanism to avoid ion accumulation in the leaves and increase salt tolerance (Munns and Tester, 2008) and a member of the high-affinity K^+ transporter (HKT) family (HKT1;5) that can take Na^+ from the root xylem into the parenchyma cells was identified as the causal gene (Gorham et al., 1990; Conde et al., 2011; Mian et al., 2011; Qiu et al., 2011). However, at higher salinity levels (200 mM NaCl instead of 150 mM NaCl) for a longer time, several genotypes accumulated Na^+ and Cl^- in plants (Figure 4). This high Na^+ concentrations interfere with K^+ uptake and K^+ function (Shabala and Cuin, 2008), and may also affect the cell wall. A number of cellulose synthase enzymes were inhibited due to displacement of Ca^{2+} by Na^+ in salt-stressed cotton (Zhong and Läuchli, 1988a). Although the Ca^{2+} contents were not significantly changed in *Miscanthus* under salt conditions, the high concentration of Na^+ may compete with Ca^{2+} and decrease activity of Ca^{2+} dependent enzymes. Cellulose synthases were also shown to be removed from the plasma membrane due to microtubule depolymerisation in *Arabidopsis thaliana* under salt stress (Endler et al., 2015). Two plant-specific proteins (Companion of Cellulose synthase 1 and 2; CC1 and CC2) were recently identified to interact with microtubules and co-localize with cellulose synthase (CesAs) to produce cellulose, and double c*c1 cc2* mutants showed hypersensitivity to salt stress but not to osmotic stress (Endler et al., 2015; Wang et al., 2016). In addition, *Arabidopsis thaliana* plants deficient in cellulose were more sensitive to salt stress (Kang et al., 2008). The cellulose content of the cell wall thus may be related to salinity tolerance, and protecting the cellulose synthesis machinery is important for maintaining cellulose content of cell walls against salt stress (Kesten et al., 2017). In the *Miscanthus* genotypes used in this study, cellulose contents in the leaves under salt stress showed genotypic variation, from 46.14 % NDF in genotype OPM-87 to 52.43 % NDF in OPM-71 (Table 6). Additionally, there was a significantly positive relationship between cellulose content and stem dry weight under salt stress (Table 7). It might therefore be worthwhile to study allelic variation and activity of genes involved in cellulose synthesis of the *Miscanthus* cell wall.

Salt stress was shown to alter lignin biosynthesis and increase the quantity of lignified xylem tissue in stems and roots of tomato and these lignified cells in vascular bundles may enhance the cell-to-cell pathway for water transport and reduce ion uptake in the cells (Sánchez-Aguayo et al., 2004). Similarly, a more intense lignification was observed in the root cells of the salt tolerant wheat species *Triticum durum* compared to the sensitive species *Triticum aestivum* (Jbir et al., 2001). We did not measure cell wall characteristics in stems, but the lignin content in the leaves of *Miscanthus* at 200 mM NaCl positively correlated with Na^+ and Cl^- ions in stems. The influence of salinity on the lignin content of the *Miscanthus* stems and the consequences for Na^+ or Cl^- transport and content needs to be further explored. There was no correlation in Na^+ contents of 6-week tillers vs 12-week tillers in the 6 tested genotypes (Supplementary Figure 1A) while there was a highly positive correlation in Na^+ contents between 12-week harvest tillers and 18-week harvest (Supplementary Figure 1 B). It is important to note that the stem Na^+ contents at 250 mM NaCl in the greenhouse for 6 weeks in the experiments described in Chapter 3 highly positive correlated with the Na^+ content of 6-week old tillers in this experiment under outdoor conditions (Supplementary Figure 1C), but not with 12-week tillers at 200 mM NaCl (Supplementary Figure 1D). This may suggest that Na^+ exclusion mechanisms are differentially regulated during the growth, or that different mechanisms apply at different stages of development. The higher ion accumulation in the older, senesced biomass than in the younger, green biomass may also be caused by the longer exposure to salt stress, resulting in higher ion accumulation in shoots. To keep low ion contents in shoots until harvest would be a preferred trait for breeding. Genotype OPM-42 showed the lowest Na^+ and Cl^- in 18-week tillers (Figure 3A and B) and 19-week whole leaf tissues (Table 4) which may be considered as a starting material for breeding for this trait. For the perennial crop *Miscanthus*, it remains to be explored what the effects are of both developmental stage and growing year under salt stress. The Na^+ contents of 18-week old tillers were as expected highly correlated with the final harvest of 19-week old whole plants. However, the ion contents in 19-week whole plants were considerably lower than the 18-week tillers. A possible explanation may be that the difference in level of senescence between 18 and 19 weeks caused the differences in ion contents, but this still needs to be further explored.

4.2 Implications for biofuel production under salt stress

Even though the twelve *Miscanthus* genotypes were able to avoid the Na^+ accumulation in the shoots at 150 mM NaCl in the pilot experiment, this salt treatment still caused osmotic stress, with changes in the cell wall compositions of stems that were similar to the changes seen in response to drought stress: cellulose content decreased, hemicellulose content increased, and lignin content nearly the same under drought stress compared to control conditions (van der Weijde et al., 2016b). This may increase the amount of structural sugars available per unit of biomass, and this effect was previously reported to increase cellulose conversion, which considerably enhances the techno-economic performance of bioconversion (van der Weijde et al., 2016b; van der Weijde et al., 2016a). In the pilot experiment, the still green biomass was harvested after 6 weeks of salt treatment. For biogas, an early green harvest delivers a higher quality with less lignin content compared with a senesced harvest, which improves saccharification efficiency (Kiesel and Lewandowski, 2017; Kiesel et al., 2017). Even if the salt stress applied in the pilot experiment may have improved the saccharification efficiency by increasing the hemicelluloses content, the total biomass was reduced by around 12% (Table 2). Therefore, there is a trade-off between the increase in conversion efficiency and the reduction of yield. If the reductions in total biomass yield per hectare associated with salt stress would be compensated by an increase in conversion efficiency during anaerobic digestion, extending cultivation to saline lands may be economically feasible. However, an early green harvest also causes a lower yield due to short vegetative growth period, and also impairs the growth of the crop in the following season because of less relocation of carbohydrates to rhizomes (Purdy et al., 2015). A pre-winter harvest for biogas utilization avoids most leaf loss and largely solves the problems of an early green harvest (Kiesel and Lewandowski, 2017; Kiesel et al., 2017; van der Weijde et al., 2017).

The pre-winter harvest of the salt stressed plants (200 mM NaCl) in this study significantly decreased biomass, cell wall contents, and hemicelluloses contents but an increase in the lignin contents compared to plants grown without stress. Low hemicellulose contents and high lignin contents in the cell walls were reported to decrease the amount of structural sugars available from biomass conversion (van der Weijde et al., 2016a; van der Weijde et al., 2017). Therefore, the relatively high salt level on average decreased not only pre-winter harvest yield but also quality in our genotypes. Genotypes with relatively high biomass and hemicelluloses contents and low lignin contents in the cell wall compositions under salt stress may be considered better candidates for producing biomass under high salinity lands (see later, section 4.4).

4.3 Useful traits for biomass quality

In our study, Na^+ and Cl^- contents in leaves showed highly negative correlations with cell wall content under salt stress (Table 7). Since biofuel yield is tightly linked to cell wall content, low leaf ion contents should be considered as an important trait for biomass. High Na^+ and Cl^- contents seem to be linked to a reduction of the total cell wall content. There are several possible explanations for this. It is possible that excess Na^+ in the apoplast interferes with cell wall synthesis, as salinity was shown to reduce glucose incorporation in cell wall polysaccharides of cotton roots (Zhong and Läuchli, 1988b). Moreover, salt stress also induces ROS, which can be scavenged by free OH groups in cell wall polysaccharides (Andrea et al., 2015; Byrt et al., 2018). This process may also interfere with cell wall synthesis. Furthermore, sugars such as fructans that also scavenge ROS, competing with their function as building blocks for cell walls (Andrea et al., 2015). Na^+ and Cl^- concentrations were also highly positively correlated with lignin contents of leaves, and this also negatively affects saccharification efficiency (van der Weijde et al., 2016a). Lignin content is a main factor for the efficiency of digesting cell wall polysaccharides into fermentable sugars (Chen and Dixon, 2007; Himmel and Picataggio, 2009; Studer et al., 2011). Although salt stress influenced cell wall composition and increased the Na^+ and Cl^- contents in the shoots, it did not change the ion contents in NDF itself; Na^+ and Cl^- contents in NDF were not significantly different between control conditions and salt stress. Therefore, most of the accumulated ions in the leaves may be intracellular, and may be stored in the vacuoles to maintain cytosolic ion homeostasis (Munns and Tester, 2008). Low ion contents in the shoots can be used as a selection criterion in breeding programs aiming for improvement of *Miscanthus* cultivation under saline conditions.

Another strategy to decrease minerals in the harvested *Miscanthus* material is a delayed harvest (Brosse et al., 2012). The K^+ and Cl^- concentrations in materials were reduced by 85-95% under normal field conditions in spring because of relocation of nutrition and minerals to rhizomes and leaching (Jorgensen, 1997). Morphological differences such as stem diameter affect the rate of leaching (Jorgensen, 1997). For example, *M. sinensis* with thin-stemmed genotypes showed more efficient leaching of minerals than *Miscanthus* × *giganteus* with thick stems (Iqbal and Lewandowski, 2014). Experiments that would include a post-winter harvest may shed light on the relocation of minerals, and effects of salinity on post-harvest quality.

The stem/leaf ratio also plays an important role in quality of the harvest under different stresses. There was a significant decrease in the stem/leaf ratio between control and drought stress (van der Weijde et al., 2016b). Similarly, salt stress significantly decreased the stem/leaf ratio from an average of 0.26 under normal condition to 0.14 under salt stress in the six tested genotypes (Figure 1B). For anaerobic digestion, low stem/leaf ratios may improve overall conversion efficiency because saccharification efficiency is higher in leaves then in stems (van der Weijde et al., 2016b; van der Weijde et al., 2017).

4.4 Potential genotypes for anaerobic digestion, combustion and breeding

The demands on biomass quality depend on the different production chains. For combustion, a high stem/leaf ratio is important, as the stem is the major harvestable part for combustion fuel. Low ion contents in the stems are also important because low ion contents prevent stoves from corroding, slagging and fouling (Brosse et al., 2012). The stem total ion contents (including Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+}) of *Miscanthus* genotypes OPM-42 and OPM-87 after 19 weeks of salt stress were 43.79 mg/g DW and 33.93 mg/g DW, respectively, and were considerably lower than the average value of 54.27 mg/g DW. There was no significant change in stem/leaf ratio of genotype OPM-42 between control conditions and salt stress, but genotype OPM-87 showed a significantly decreased stem/leaf ratio from 0.28 under normal conditions to 0.09 under salt stress (Table 3). Genotype OPM-42 was also the highest plant of the 6 genotypes at 200 mM NaCl. In addition, this genotype under salt stress showed a slight increase in cell wall contents and cellulose contents compared with control conditions. Therefore, genotype OPM-42 has the highest potential as feedstock for combustion and as a value breeding resource. On the other hand, low stem/ leaf ratio, and easier digestibility (high hemicelluloses and low lignin content) are important traits for saccharification efficiency during anaerobic digestion (van der Weijde et al., 2017; Wagner et al., 2017). Genotype OPM-48 and OPM-87 showed relatively high hemicellulose contents and low lignin contents under salt stress (Table 6). In addition, these two genotypes also showed significant decreases in the stem/leaf ratios under salt stress (Table 3). For biogas production using biomass produced under saline conditions, these two genotypes are better candidates.

The considerable variation in cell wall composition that we observed between six genotypes under control and salt stress suggests that the genetic diversity for these traits in the *Miscanthus* germplasm pool is even broader. Exploring this variation will support the identification of genotypes with even higher potential to design optimal varieties for different biofuel applications on marginal lands.

5 Funding

The research received funding from the European Union consortium OPTIMISC (project ID 289159). Chang-Lin Chen is supported by a scholarship from the Ministry of Education, Taiwan.

6 Acknowledgments

This work was performed as part of the OPTIMISC project, which received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement No. 289159. The authors gratefully acknowledge expert advice from Oene Dolstra on choice of materials, and the expert assistance with the cultivation of Geurt Versteeg and Unifarm.

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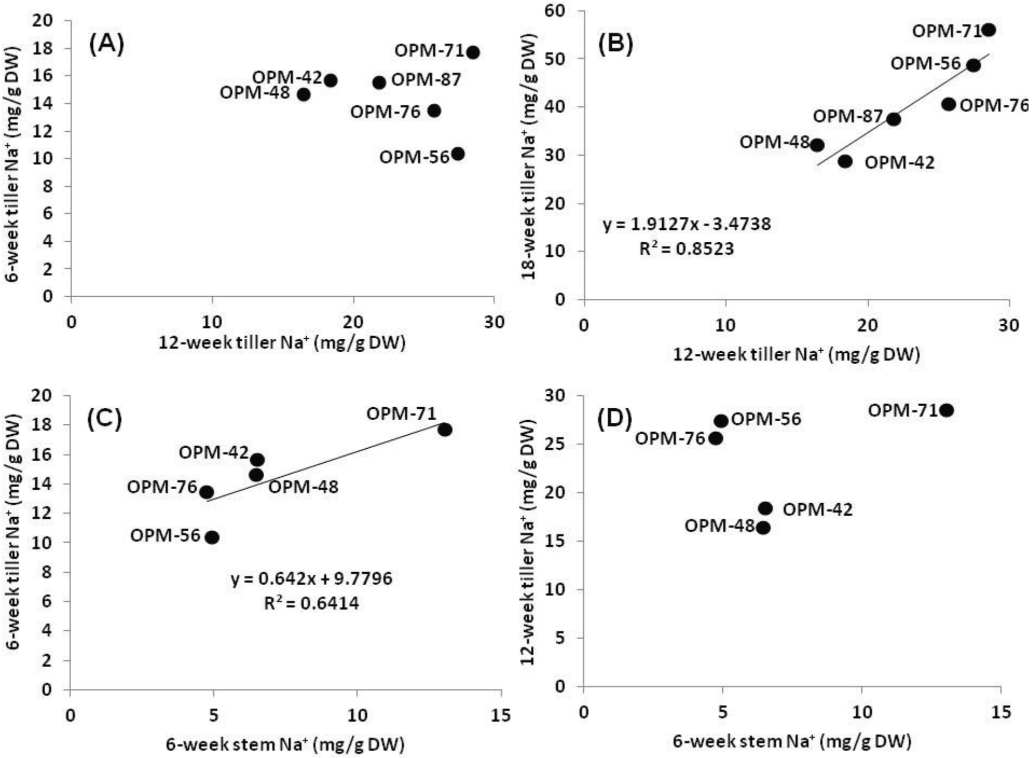
Supplementary material



Supplementary Figure 1. The relationship of tiller Na^+ of 6 genotypes at 200 mM NaCl between 6 weeks and 12 weeks (A) and between 12 weeks and 18 weeks (B). The relationship of 5 genotypes between stem Na^+ at 250 mM NaCl in greenhouse for 6 week and tiller Na^+ at 200 mM NaCl outdoor conditions for 6 weeks (C) and 12 weeks (D).

Chapter 5

Field evaluation for salt tolerance of *Miscanthus* genotypes

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Keywords: *Miscanthus*, salt tolerance, osmotic stress, ionic stress, accession

Abstract

Miscanthus is a perennial rhizomatous C4 grass with a large genetic diversity that has high potential for producing biomass under stressful conditions on marginal lands. Salt tolerant and high-yielding *Miscanthus* genotypes are required for optimizing biomass production on saline soils. Twenty accessions (which were collected from various locations in China) and five genotypes (OPM-1, 3, 6, 8, and 9) from Aberystwyth University, UK were evaluated under marginal saline field conditions in Dongying, China. The *Miscanthus* genotypes/accessions showed high genetic variation in salt tolerance in this field trial. On average, the *Miscanthus* genotypes had 54% reduction in yield at EC 6 dS/m in the second growing year (one year after establishment). At this salinity level, several collected accessions form Dongying (CN32, 33, 34, 35, and, 36) had the highest yield in both control and salt stress conditions, and the accession CN4 from Haizhou had the highest salt tolerance. Accession CN32 had the highest yield at a salt concentration below EC 2.5 dS/m, and may be planted on relatively mild saline soils, while the accessions CN14 and CN34 with relatively high salt tolerance and yield at EC 10 dS/m were recommended to be grown on lands with higher salinity levels. For biomass production under moderately saline conditions, accession CN36 was the most promising candidate with the highest yields at EC 6 dS/m. In addition to shoot biomass, the stem-to-leaf ratio is important for combustion. Five accessions (CN31, 33, 34, 35 and 36) had a stem/leaf ratio that was more than 2 at EC 6 dS/m, and four accessions (CN31, 34, 35, and 36) maintained a ratio above 1.7 at EC 10 dS/m. Accession CN34 may therefore be considered as both a breeding parent to improve salt tolerance in general and as planting material for producing biomass under high salinity, because of its superior quality and biomass yield under these highly saline conditions.

1 Introduction

Soil salinity is one of the main problems for agriculture as it decreases crop yield and quality. Almost 20% of the world's irrigated land is adversely influenced by salinity (Munns and Tester, 2008) and soil salinity is further increasing due to poor drainage and climatic change (Bennett and Khush, 2003). Saline soils are classified by solute electrical conductivity (EC) in deci-Siemens/meter (dS/m) as: non-saline (<2 dS/m), slightly saline (2-4 dS/m), moderately saline (4-8 dS/m), strongly saline (8-16 dS/m), and very strongly saline (>16 dS/m) (Rhoades, 1999). Salinity affects plant growth and development due to osmotic stress, ionic stress, and by causing nutritional imbalance (Ashraf and Harris, 2004; Munns and Tester, 2008). Osmotic stress affects growth immediately because of the direct effects of a high concentration of ions surrounding the roots and the responses of leaves are similar to the symptoms under drought stress (Munné-Bosch et al., 2001). Ionic stress develops over time due to ion accumulation within the shoots. The specific effects on leaf senescence have been related to the accumulation of Na^+ or Cl^- ions; salt sensitive wheat genotypes senesced earlier and had higher ion concentrations in the leaves than salt tolerant genotypes (Husain et al., 2003; Munns et al., 2006). Although both ionic exclusion and osmotic tolerance are involved in salt tolerance, it is possible that some tolerance mechanisms are more effective under particular circumstances. Osmotic tolerance may be more important under moderately saline conditions (EC 4-8 dS/m) and ion exclusion tends to be more effective in conditions of higher salinity levels (EC 8-16 dS/m) (Munns et al., 2012; Roy et al., 2014).

Miscanthus is a genus of high-yielding perennial rhizomatous grasses with C4 photosynthesis that originates from Southeast Asia, the Pacific islands, and tropical Africa. It is considered as a promising candidate biofuel crop and multiple biomass use options due to high yields, low input demands, and the potential to be cultivated on marginal lands thus avoiding competition with agricultural lands needed for food production (Zub and Brancourt-Hulmel, 2010). Combustion is the most common utilization option with higher energy yield than biogas, but biogas is considered as an alternative because it is easy to transport and store (Kiesel et al., 2017). *Miscanthus* × *giganteus* is the genotype that is commercially used for biomass production due to several advantageous characteristics such as high yield and quality for combustion, but it has some serious disadvantages. Firstly, *Miscanthus* × *giganteus* as a triploid hybrid is propagated by rhizomes or tissue culture, which increases costs for planting compared to seed propagation and direct seeding (Xue et al., 2015; Clifton-Brown et al., 2017). Secondly, the biomass yield of *Miscanthus* × *giganteus* is affected by abiotic stress conditions. It is more susceptible to frost and drought stress as compared with some genotypes of *M. sinensis* (Hastings et al., 2009a; Hastings et al., 2009b; Fonteyne et al., 2016). Thirdly, extending cultivation further to marginal areas requires breeding programs aimed at stress-tolerant genotypes, but *Miscanthus* × *giganteus* (3n =3x =57) cannot be directly used in breeding as it is a sterile triploid. Whereas it is difficult to overcome these limitations in *Miscanthus* × *giganteus*, the parental species for *Miscanthus* × *giganteus, M. sinensis* (2n =2x =38) and *M. sacchariflorus* (2n =4x =76), can be crossed and used to improve stress tolerance. Tetraploid *M. sacchariflorus* and species hybrids tend to have thicker and longer stems than *M. sinensis*, leading to higher yields in trials (Iqbal and Lewandowski, 2014), but *M. sinensis* is more amenable to breeding as it is diploid.

Besides high yield, quality of the biomass for bioenergy purposes is another important factor. There are several ways to improve biomass composition and enhance combustion quality. Firstly, delaying harvesting time can reduce the plant's level of mineral contents that cause corrosion and fouling during combustion (Baxter et al., 2012, 2014). Secondly, using early flowering genotypes is another option. Genotypes with early flowering initiate relocation of nutrients earlier and complete it before winter frost kills the stems (Jensen et al., 2017). This not only decreases the nutrients in the shoots and increases combustion quality but also saves fertilizer inputs for growing (Smith and Slater, 2011; Iqbal et al., 2017). Thirdly, a high stem-to-leaf ratio plays a key role in determining biomass quality for combustion because most minerals accumulate in leaves under normal field conditions.

As a second generation bioenergy crop, *Miscanthus* is expected to be grown on marginal lands like saline soils in order to avoid competition with food production on fertile lands. Most research has focused on salt tolerance in the commercial *Miscanthus* genotype *Miscanthus* × *giganteus*, using rhizome based plantlets under greenhouse conditions for short periods of time (Plazek et al., 2014; Stavridou et al., 2016), and we extended these studies to include other hybrids, *M. sacchariflorus* and *M. sinensis* genotypes. However, it is still unknown whether seeded hybrids can survive on saline lands for the first year and produce stable biomass from the second and following years on saline lands. Moreover, traits that affect quality of the biomass, like ion content and stem-to-leaf ratio, are still unexplored in *Miscanthus* when grown in fields under salt stress.

Although it is a challenge to produce stable amounts of biomass of high quality at an economical viable scale on saline lands, there have been some successful examples of salt stress improvement in other crops, like wheat and barley (Nevo, 2014). *Triticum dicoccoides* and *Hordeum spontaneum*, the progenitors of cultivated wheat and barley, respectively, have adapted to a broad range of environments and show rich genetic diversity for drought and salt tolerance (Nevo et al., 1986; Nevo and Chen, 2010; Nevo, 2014). Superior genotypes were identified even under high salinity (EC= 33 dS/m) from five selected populations of *H. spontaneum* collected from the Mediterranean Coastal Plain and the northern Negev desert. Likewise, superior genotypes from five populations of *T. dicoccoides* from the eastern Samaria steppes, Mt. Hermon and Mt. Carmel can reach full maturity in saline soils (EC= 24 dS/m) (Nevo et al., 1993). Genes and quantitative trait loci (QTLs) conferring drought and salt tolerance have been identified in *T. dicoccoides* and *H. spontaneum* and have been transferred to wheat and barley cultivars, respectively (Nevo, 2014). Similarly, *Miscanthus* shows a wide range of tolerance against abiotic stress (Quinn et al., 2015), likely related to its occurrence as natural vegetation in regions ranging from mountains to coastal areas. By introducing new germplasm from wild collections, it should be possible to overcome abiotic stresses such as cold, drought and salinity, and extend the geographical range for producing biomass (Lewandowski et al., 2016). Therefore, it is conceivable that salt tolerant *Miscanthus* genotypes from harsh environments can be identified and potentially utilized as breeding materials. Because salt stress includes an osmotic stress component, salt tolerance may be associated with drought tolerance. Therefore, we collected for this study new *Miscanthus* accessions in dry areas of the west China as well as coastal regions, and evaluated the accessions in a field trial on saline soil in Dongying, China. This field trial also provided an opportunity to evaluate whether plants derived from seeds (as opposed to plants from rhizomes) can be planted and grown successfully on marginal lands and give a respectable economic yield. In addition to the 20 accessions, five well-performing genotypes with rhizomes from the OPTIMISC project (including *Miscanthus* × *giganteus* as the reference genotype) (Kalinina et al., 2017) were included in this trial for comparison. The performance and response to salinity of the genotypes was evaluated on marginal lands, including a low nutrition, no irrigation system, and at different levels of salinity. Based on phenotypic data under different salt levels, Chinese accession CN34 demonstrated not only relatively high yields and salt tolerance but also high stem-to-leaf ratios at EC 10 dS/m. Therefore, it should be considered as a high potential resource for growth on saline soils and as valuable starting and breeding material for improvement of salt tolerance of *Miscanthus*.

2 Materials and Methods

2.1 Plant materials

The twenty-five genotypes/accessions of *Miscanthus* used for evaluation are listed in Table 1. This set included 20 *M. sacchariflorus* accessions, 2 *M. sacchariflorus* genotypes (OPM-1 and 3) and 3 hybrids (OPM-9: *Miscanthus* × *giganteus*, OPM-6: *M. sacchariflorus* × *M. sinensis*, and OPM-8: *M. sacchariflorus* × *M. sinensis*). The Chinese *M. sacchariflorus* accessions collected from different locations in China in 2012 (Figure 1) had a CN code. These accessions contain potentially multiple genotypes, as these are open-pollinated seeds. Genotypes OPM-1, 3, 6, 8, and 9 propagated from rhizomes with the same genetic background were considered as genotypes. The seeds were germinated and grown in a greenhouse for about 2 months to obtain seedlings of about 5-10 cm height in 2013. The five OPM genotypes were propagated from rhizomes in a glasshouse to obtain rhizome-derived plantlets. Both seedlings and rhizome-derived plantlets were transplanted into the field on 16th to 20th June 2013.

Figure 1. The Chinese *M. sacchariflorus* accessions were collected from the indicated locations in China in 2012. This figure was adepted from d-maps.com.

Table 1. Information on the source species for all 25 accessions/genotypes and on the habitat in which germplasm was collected for the 20 CN accessions of *Miscanthus* used in this field trial.

Genotype /Accession	Species	Supplier	Collecting location	Habitat	Germplasm type
OPM-1	*M. sacchariflorus*	IBERS			Rhizome
OPM-3	*M. sacchariflorus*	IBERS			Rhizome
OPM-6	*(M. sacchariflorus × M. sinesnsis)*	IBERS			Rhizome
OPM-8	*(M. sacchariflorus × M. sinesnsis)*	IBERS			Rhizome
OPM-9	*Miscanthus × giganteus*	IBERS			Rhizome
CN4	*M. sacchariflorus*	DAI	Haizhou	Roadside	Seed
CN8	*M. sacchariflorus*	DAI	Xi'an Chanhe	Riverside	Seed
CN9	*M. sacchariflorus*	DAI	Xi'an Chanhe	Riverside	Seed
CN10	*M. sacchariflorus*	DAI	Xi'an Chanhe	Riverside	Seed
CN12	*M. sacchariflorus*	DAI	Xi'an-Bailuyuan	Highland roadside	Seed
CN13	*M. sacchariflorus*	DAI	Dongtai	Coastal roadside	Seed
CN14	*M. sacchariflorus*	DAI	Dongtai	Coastal roadside	Seed
CN16	*M. sacchariflorus*	DAI	Dongtai	Coastal roadside	Seed
CN18	*M. sacchariflorus*	DAI	Dongtai	Coastal roadside	Seed
CN23	*M. sacchariflorus*	DAI	Dongtai	Coastal roadside	Seed
CN27	*M. sacchariflorus*	DAI	Nanjing	Fieldside	Seed
CN28	*M. sacchariflorus*	DAI	Nanjing	Fieldside	Seed
CN29	*M. sacchariflorus*	DAI	Nanjing	Fieldside	Seed
CN30	*M. sacchariflorus*	DAI	Nanjing	Fieldside	Seed
CN31	*M. sacchariflorus*	DAI	Nanjing	Fieldside	Seed
CN32	*M. sacchariflorus*	DAI	Dongying	Coastal wetland	Seed
CN33	*M. sacchariflorus*	DAI	Dongying	Coastal wetland	Seed
CN34	*M. sacchariflorus*	DAI	Dongying	Coastal wetland	Seed
CN35	*M. sacchariflorus*	DAI	Dongying	Coastal wetland	Seed
CN36	*M. sacchariflorus*	DAI	Dongying	Coastal wetland	Seed

(IBERS) Institute of Biological Environmental and Rural Sciences, Aberystwyth University, UK. (DAI) Dongying Agricultural Institute, Dongying, China.

2.2 Trial management and climatic conditions

The field trial was arranged at two sites (site A and site B) at the New Agricultural Demonstration Area of Dongying, Shandong Province, China, about 20 kilometers from the Bohai Sea. The two sites were 500 meters apart. The soil EC value in site A varied from 1.70 to 10.31 dS/m, while the EC value in site B varied from 2.89 to 22.64 dS/m, measured in soil samples taken prior to planting (measured in a 1:2 soil:water dilution, and recalculated to soil paste EC as described in section 2.3). No fertilizer and irrigation were applied at both site A and B to create natural marginal conditions. The trial was designed as a split plot, with random block arrangement at both sites. Field plans of plots and blocks are shown in Supplenmentary Figure 1. Each plot occupied 4 square meters and a 1-meter bare row was located between plots. There were 8 seedlings/plantlets planted in each plot, at a density of 2 plants/m^2. After planting, the remaining seedlings were mixed and grown as border rows surrounding each trial site. Climate data (rainfall and air temperature) were obtained from the weather stations near the study sites (https://rp5.ru/). Supplementary Figure 2 summarizes the climatic conditions during two consecutive growing seasons.

2.3 Soil sampling and analysis

Soil samples were taken from the middle of each plot each year and analyzed for soil EC values. The soil samples were collected on 23rd May 2013 and 15th June 2014. For measuring the EC value, 20 grams of dried soil was mixed with 40 ml de-ionized water in a 250-ml beaker. After stirring thoroughly, the samples were allowed to equilibrate for 1 hour. The suspension was filtered through filter paper #2. Subsequently, the solution was measured using the Elmeco Electrical Conductivity (EC) meter, Tasseron (Netherlands). Measurement of electrical conductivity of 1:2 soil to water extract ($EC_{1:2}$) is simple and fast but the influence of salinity on plant growth is more often expressed as electrical conductivity of saturated paste extract (EC). Therefore, we convert $EC_{1:2}$ to EC in order to assess plant response using the formula: $EC = 5.3\ EC_{1:2}$ (ILRI, 2003; Khorsandi and Yazdi, 2011). The relationship of plot EC value in 2013 and 2014 is shown in Supplementary Figure 3. The plot EC value and Na^+ and Cl^- content of the solution (1:2 soil to water extract) in 2014 were strongly correlated, indicating NaCl contents in the soil largely determines the variation in the soil EC (Supplementary Figure 4).

2.4 Plant sampling and measurement

Plants were harvested when most plants showed the first signs of senescence, which was from 26th to 30th October in 2013 as well as in 2014. From each plot, shoots from 8 plants were cut, divided into leaves and stems, after which fresh biomass weight was determined for leaves and stems. After 2 weeks of air-drying in a well-ventilated room, dry weight was determined for both leaves and stems. The number of tillers in each plot were counted and were used to estimate the plot yield.

2.5 Ion chromatography

For determination of the ion contents in the leaves and stems of each genotype, each sample from each plot was ground to a fine powder using a hammer mill with a 1 mm sieve. Dry powdered samples (25-30mg) were ashed at 575°C for 5 hours. Ashed samples were dissolved by shaking for 30 minutes in 1 ml 3M formic acid at 99°C and then diluted with 9 ml Milli-Q water. The samples were shaken again at 80°C for another 30 minutes. A final 500x dilution was subsequently prepared by mixing 0.2 ml sample solution with 9.8ml Milli-Q to assess the Na^+, K^+, Cl^-, Mg^{2+}, and Ca^{2+} content of each leaf and stem sample using the Ion Chromatography (IC) system 850 Professional, Metrohm (Switzerland).

2.6 Statistical analysis

Because of the high variability of salinity across the field sites, experimental data were used to derive an equation that could be used to estimate the yield at the different levels of EC. The actual shoot dry weight and stem dry weight at the different levels of EC were modeled through fitting exponential curves (Eqn 1) or logistic curves (Eqn 2) to the data of individual genotypes/accessions. In Eqn (1) and Eqn (2), Y_{DW} is dry weight of shoots or stems per square meter, b_0 and b_1 are the constants for different genotypes/accessions, and S is the EC value for each plot. The equation for each genotype/ accession estimating the yield under different EC values is listed in Supplementary Table 1 and two examples are shown in Supplementary Figure 5. The average yield of each genotype/accession below an EC 2.5 dS/m was calculated directly from the field data at low EC levels.

$$Y_{DW} = b_0 \times e^{b_1 \times s} \tag{1}$$

$$Y_{DW} = b_0 + b_1 \times ln(s) \tag{2}$$

The ion content of leaves and stems from different saline conditions were classified into 4 groups according to EC values (0-2, 2-4, 4-6 and >6) and compared by analysis of variance. All statistical analyses were performed using the statistical software package Genstat 15th edition (VSN International Hemel Hempstead, UK).

3 Results

3.1 Overview of plant performance and collection locations

The EC values in the field ranged from 1.70 dS/m to 22.64 dS/m in 2013 and from 1.78 dS/m to 30.11 dS/m in 2014. The average of the EC values over the plots increased from 6.47 dS/m in 2013 to 7.71 dS/m in 2014. During this study, some plots were flooded between 2013 and 2014 which caused sharp changes in the EC values of these plots (see Supplementary Figure 3). The plots with too large variation of the EC values between the 2 years were discarded and not considered in the performance. This reduced the number of replications of two genotypes (OPM-3 and OPM-6) and one accession (CN23) too much to get reliable estimates of their yield using the curve-fitting procedure. Genotype OPM-9 only survived below EC 2.65 dS/m, which also did not leave enough data for yield estimates. Therefore, only twenty-one genotypes/accessions could be analyzed for biomass production under different salinity levels in the two growing seasons.

The shoot dry weight was significantly ($P < 0.001$) influenced by the EC value. The average yield reduction of the twenty-one genotypes/accessions is shown in Figure 2. When the soil EC value increased from 2 to 4 dS/m, the average reduction was 40 % in the first year and 34.6 % in the second year. At EC values of 6 dS/m, the mean of yield reduction across all genotypes/accessions was 64% in 2013 and 54% in 2014 and at 10 dS/m, the yield reduction was 87% in 2013 and 79% in 2014, and the plants were seriously damaged. We chose an EC value of 6 dS/m (moderately saline) to identify salt tolerant genotypes/accessions. The 19 seeded accessions were classified into 5 groups based on their collection locations, and the 2 rhizome-derived genotypes OPM-1 and 8 were placed in a separate sixth group (Figure 3). The accessions from Dongying had highest average yield under both control and salt stress conditions and the rhizome-derived genotypes (OPM-1 and 8) demonstrated the lowest yield under both control and salt conditions (Figure 3). The accessions from Haizhou were most salt tolerant, and genotypes of the OPM group had the lowest salt tolerance. The accessions collected from Nanjing had the lowest yields of the CN groups under both control and salt conditions but showed 45% salt tolerance, which was significantly higher than the yield of the OPM genotypes (29%) at EC 6 dS/m. The relative performance of these six groups in 2013 was comparable to 2014 (not shown).

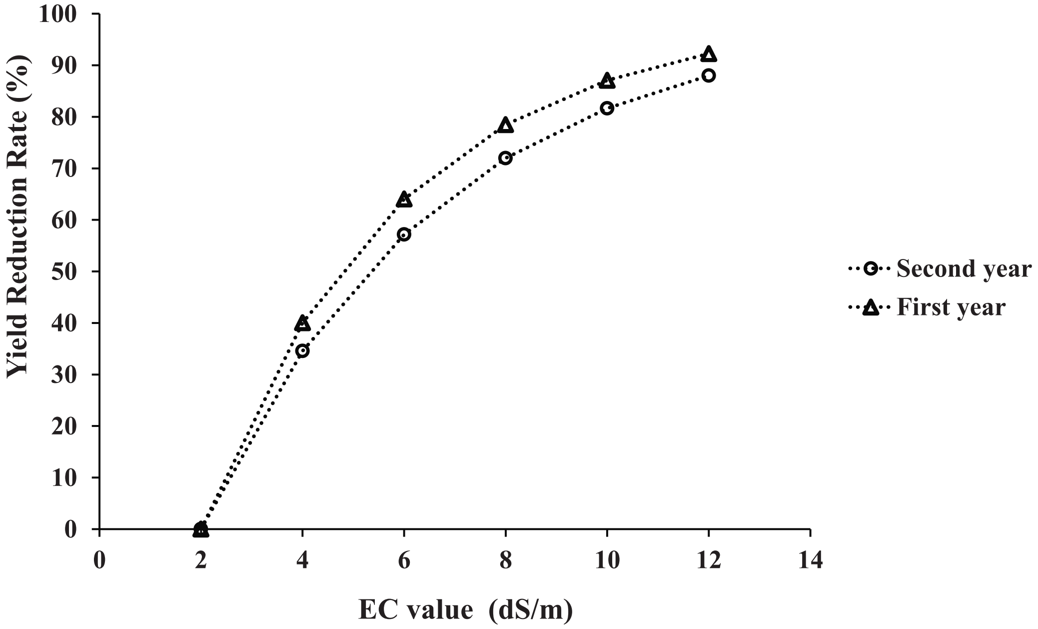

Figure 2. Yield reduction rate on different soil EC (dS/m) values in the first year (2013) and second year (2014).

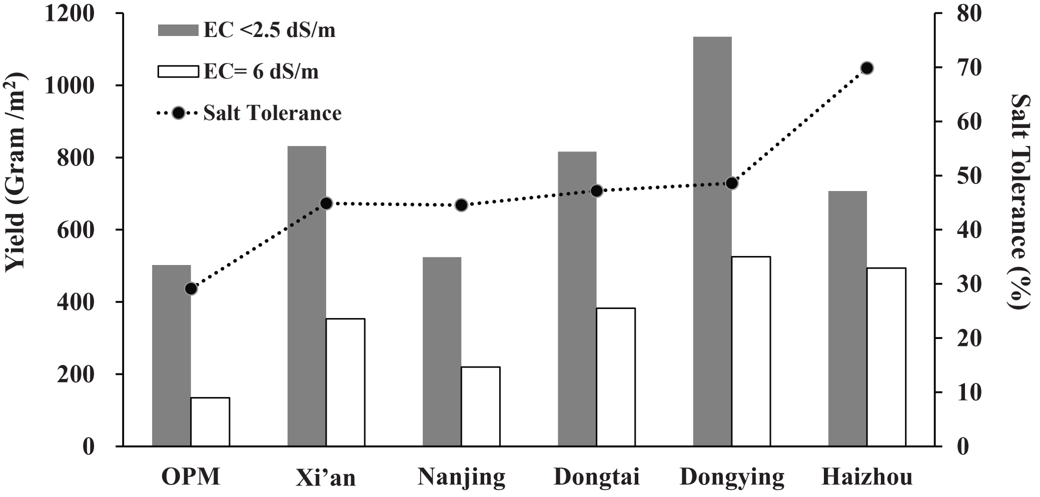

Figure 3. Shoot dry weight (bars) and salt tolerance (line-scatter plot, calculated as the ratio of shoot dry weight) at 6 dS/m (white bars) and shoot dry weight below 2.5 dS/m (gray bars) of 5 groups based on seeded seedlings from the different collection locations, plus the rhizome-derived genotypes from IBER*S* as another group, grown in the field in 2014.

3.2 Shoot dry weight and salt tolerance

Salt tolerance (ST) was assessed as the percentage of shoot biomass production under salinity relative to control conditions. The average salt tolerance of the 21 tested genotypes/accessions at an EC value of 6 dS/m was 46% in 2014. At this salt level, the genotypes/accessions showed large variation for salt tolerance, from 16% for OPM-1 to 70% for CN4 (Table 2). The five most salt tolerant accessions were CN4, CN27, CN34, CN8, and CN28 with 70, 67, 66, 62, and 61% ST, respectively at EC 6 dS/m. At this EC value, accession CN36 had the highest estimated yield with 600 g/m^2, followed by CN35, CN33, CN34, CN4, and CN32 with 525, 525, 504, 494, and 471 g/m^2, respectively. The accessions CN34 and CN4 combined high salt tolerance with high yield. Accession CN36 produced the highest yield (600 g/m^2) in combination with a medium (slightly more than average) salt tolerance (53%) at EC 6 dS/m. At EC levels with little or no salinity stress (EC 2.5 dS/m), accession CN32 had the highest yield (1386 g/m^2) but this sharply decreased to 471 g/m^2 at EC 6 dS/m. Three other accessions (CN36, CN33, and CN35) demonstrated yields of more than 1000 g/m^2 below EC 2.5 dS/m. Therefore, these accessions may have high potential to produce high yield in this environment with less salt stress. When the EC value increased to 10 dS/m, accession CN34 showed the highest yield with 302 g/m^2 and the second highest salt tolerance with 39%, and accession CN27 had less yield with 182 g/m^2 but the highest salt tolerance with 43% (Table 2). Consequently, these two accessions could be recommended for cultivation under highly saline marginal conditions. In 2013, accessions CN4, 14, 28, 32, 33, and 35 had relatively higher yield than others at EC 2-3 dS/m, and CN14, 35, and 36 showed high yield under moderately saline conditions (4-8 dS/m). Accessions CN4, CN14, CN 32, CN33, and CN36 also showed relatively high yields at EC 6 dS/m in 2014.

Table 2. The estimated shoot dry weight (SDW) and salt tolerance (ST) at four EC values in 2014.

Genotype	EC <2.5 dS/m	EC =6 dS/m		EC =10 dS/m	
	SDW (g/m^2)	SDW (g/m^2)	ST (%)	SDW (g/m^2)	ST (%)
CN4	707	494	70	209	30
CN8	575	355	62	125	22
CN9	983	220	22	56	6
CN10	965	428	44	241	25
CN12	803	410	51	162	20
CN13	746	449	60	194	26
CN14	805	413	51	284	35
CN16	971	396	41	168	17
CN18	743	271	36	91	12
CN27	427	288	67	182	43
CN28	436	266	61	156	36
CN29	480	158	33	71	15
CN30	679	156	23	28	4
CN31	597	229	38	93	16
CN32	1386	471	34	94	7
CN33	1381	525	38	237	17
CN34	765	504	66	302	39
CN35	1005	525	52	238	24
CN36	1136	600	53	273	24
OPM-1	593	96	16	14	2
OPM-8	412	173	42	84	20
Average	790	354	46	157	21

3.3 Stem dry weight and stem to leaf ratio

Stem dry weight is the major yield component for combustion purposes after a winter or spring harvest. Accession CN33 showed the highest stem dry yield with 1102 g/m^2 below 2.5 dS/m, followed by CN32, CN36, CN35, and CN10 with 992, 905, 748, and 671 g/m^2, respectively in 2014 (Table 3). The average reduction of stem dry weight was 63% at EC 6 dS/m, with the top five accessions with respect to stem dry weight being CN36, CN35, CN33, CN34, and CN4 with 434, 371, 361, 340, and 289 g/m^2, respectively. At EC 10 dS/m, only accession CN34 and CN36 were able to produce more than 170 g/m^2 (overall average 74 g/m^2). For the yield in 2013, the top five yield in non-salinity were accession CN35, 36, 32, 33, and 31 and accession CN36 had relatively high stem dry weight in moderately salinity. Therefore, several accessions with good performance and high yield in the first year may also have a high change to produce higher than others in the seconded year. A stable and high stem/leaf ratio under salt stress is considered as an important trait in *Miscanthus* for combustion. The average stem/leaf ratio was strongly influenced by EC values (Table 3) and decreased from 2.03 below EC 2.5 dS/m to 1.31 at EC 6 dS/m, falling further to 0.94 at EC 10 dS/m. The stem/leaf ratio showed high variation, from 3.95 for CN33 to 0.77 for CN29 below EC 2.5 dS/m, and at EC =6 dS/m this changed to the highest value of 2.61 for CN36 and lowest of 0.53 for CN29. At EC 6 dS/m, there were five accessions (CN31, 33, 34, 35, and 36) with a stem/leaf ratio higher than 2. When the EC value increased to 10 dS/m, accession CN31 had the highest stem/leaf ratio with 2.10. The best accessions after CN31 were 3 accessions (CN34, 35, and 36) that maintained stem-to-leaf ratios of around 1.7. Although accession CN31 showed the highest ratio of stem/leaf at high salt stress, its stem biomass was lower than the overall average under both control (520 g/m^2) and salt conditions (195, and 74 g/ m^2, respectively). However, with its potential to keep a high stem ratio under salt stress, it could be considered as a valuable breeding material. Accessions CN33, 34, 35, and 36 had higher yields than the average under moderate to strong salt levels (EC 3-10 dS/m). The largest reduction of stem-to-leaf ratio was observed in accession CN32 that changed from 2.52 below EC 2.5 dS/m to 0.42 at EC 10 dS/m. In other words, this accession became leafier and produced proportionately less stems under salt stress. Accession CN32 may therefore suit non-saline to slightly saline lands while accession CN36 and CN34 were better performers under moderately saline and strongly saline, respectively.

Table 3. Estimated stem dry weight and stem /leaf ratio at four different EC levels (2014 field experiment).

Genotype	Stem dry weight (g/m^2)			Stem/leaf ratio		
	EC <2.5 dS/m	EC =6 dS/m	EC =10 dS/m	EC <2.5 dS/m	EC =6 dS/m	EC =10 dS/m
CN4	445	289	101	1.70	1.41	0.94
CN8	312	178	46	1.19	1.01	0.58
CN9	611	92	16	1.64	0.72	0.40
CN10	671	215	102	2.28	1.01	0.73
CN12	447	188	59	1.26	0.85	0.57
CN13	400	202	73	1.15	0.82	0.60
CN14	435	180	105	1.17	0.77	0.59
CN16	512	169	57	1.11	0.74	0.51
CN18	484	116	26	1.87	0.75	0.40
CN27	209	118	63	0.96	0.69	0.53
CN28	262	125	58	1.50	0.89	0.59
CN29	208	55	23	0.77	0.53	0.48
CN30	456	100	17	2.04	1.79	1.55
CN31	444	163	63	2.91	2.47	2.10
CN32	992	242	28	2.52	1.06	0.42
CN33	1102	361	138	3.95	2.20	1.39
CN34	604	340	190	3.75	2.07	1.70
CN35	748	371	150	2.92	2.41	1.70
CN36	905	434	176	3.92	2.61	1.81
OPM-1	425	59	7	2.53	1.59	1.00
OPM-8	250	102	46	1.54	1.44	1.21
Average	520	195	74	2.03	1.33	0.94

3.4 Ion content in *Miscanthus* tissues at different salt levels

The ion contents of leaves and stems at different EC values are listed in Table 4 (for this, EC-values of plots were used to make bins, and ion contents of accessions/genotypes within an EC range were averaged). Generally, the ion contents in the stems were lower than those in the leaves under both control and salt conditions. The leaf ion contents for Na^+, K^+, Ca^{2+} and Cl^- did not show significant differences between EC 0-2 dS/m, EC 2-4 dS/m and EC 4-6 dS/m, but were significantly increased for Na^+, Mg^{2+} and Cl^- above 6 dS/m. However, the leaf K^+ and Ca^{2+} contents at this EC level were not significantly different. Similarly, stem Na^+, Mg^{2+}, and Cl^- contents showed a significant ($P<0.001$)

increase above EC 6 dS/m compared to EC values in the range of 0-6 dS/m. Remarkably, the stem K^+ contents also increased significantly from 2.87 mg/g DW at EC values in the range of 0-2 dS/m to 8.02 mg/g DW above EC value of 6 dS/m. There was no significant difference in Ca^{2+} contents in the stems at different EC values.

Table 4. The contents of Na^+, Cl^-, Ca^{2+}, Mg^{2+}, and K^+ in leaves and stems in 2014.

EC range (dS/m)	Leaf (mg/g DW)					Stem (mg/g DW)				
	Cl^-	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	Na^+	K^+	Ca^{2+}	Mg^{2+}
0-2	0.98 a	2.02 a	2.97 a	6.67 a	3.02 a	0.00 a	1.59 a	2.87 a	1.04 a	1.12 a
2-4	1.50 a	2.73 a	3.25 a	7.55 a	3.30 a	0.20 a	2.29 a	3.17 a	2.52 a	1.09 a
4-6	1.12 a	2.50 a	2.81 a	8.39 a	4.46 b	0.21 a	3.48 a	3.96 a	1.77 a	1.66 b
>6	4.00 b	5.74 b	3.19 a	8.32 a	5.48 c	4.22 b	7.21 b	8.02 b	3.50 a	2.68 c

The means marked with the same letters did not show significant differences in the different EC ranges (multiple range Fisher's test, $p < 0.05$).

4 Discussion

Bioenergy crops could be an important alternative for fossil fuel and *Miscanthus* has been considered as a promising bioenergy crop in Europe since the 1980s (Brosse et al., 2012; Chung and Kim, 2012). In order to become a successful second generation bioenergy crop, *Miscanthus* is preferably planted on underutilized marginal lands like saline soils to avoid competition with food crops to be grown on agricultural lands. The most important for cultivation on marginal lands at an economical scale is to develop suitable genotypes that are able to maintain a stable and high-quality biomass production under stress conditions as well (Lewandowski et al., 2016), and for lands that are saline, it is essential to develop salt tolerant genotypes. Most research up to now has focused on the commercial genotype *Miscanthus* × *giganteus,* which was assessed for yield and quality only for short stress periods under greenhouse conditions (Plazek et al., 2014; Stavridou et al., 2016). In order to produce economically biomass from perennial bioenergy crops such as *Miscanthus*, there is an additional need to develop low-cost hybrid seed production strategies for planting the crop and to reach maximum yield in the second or third year of planting (Clifton-Brown et al., 2017). This chapter presents the evaluation of 21 genotypes, for the larger part consisting of *M. sacchariflorus* accessions collected in China, for growth under marginal saline conditions. Clifton-Brown et al. (2001) showed in their evaluation of fifteen *Miscanthus* genotypes at five sites in Europe during the first three growing seasons that the third-year and second-year yields were highly correlated, while the third-year and first-year yields had a lower correlation (0.81 vs 0.56). Our evaluation identified several *Miscanthus* accessions as promising candidates both for breeding purposes and for cultivation on marginal saline lands.

4.1 Reduction of biomass under salt stress and salt tolerance in different populations

Several studies have addressed salt tolerance of *Miscanthus* under controlled conditions, but multi-year salt stress tolerance evaluations under field conditions in this perennial crop still remains to be done. Before being able to extend *Miscanthus* cultivation to marginal lands, more data from field trials needs to be collected to understand whether the production on marginal lands will be economically feasible. In a salt tolerance study in the greenhouse, young *M. sinensis* plants grown from seeds demonstrated 50% reduction in biomass at EC 10.5 dS/m (Sun et al., 2014). In another study, *Miscanthus* × *giganteus* grown from rhizomes in pots under greenhouse conditions showed a significant decline in shoot dry weight (50%) at approximately 10.6 dS/m (120 mM NaCl) (Stavridou et al., 2016). Extending the results from greenhouse conditions to fields by modelling the reduction of shoot dry weight in *Miscanthus* × *giganteus* showed nearly 50% yield reduction in the range of EC 3-4.99 dS/m in European regions (Stavridou et al., 2016). However, in our field trial the same genotype OPM-9 (*Miscanthus* × *giganteus*) only survived below an EC value of 2.65 dS/m. The average biomass reduction across all genotypes/accessions amounted to around 54 % at 6 dS/m in the second year (2014). It is important to mention that the field trial site at Dongying for our study was most likely also poor in nutrients, as opposed to the experiments mentioned above which used 0.5X Hoagland nutrition with different salt levels under controlled conditions. At this marginal field site, plants may be more seriously damaged due to a combination of different stresses, i.e. drought stress and nutrient limitation (Supplementary Table 2) in addition to the salt stress. There was an uneven distribution of rainfall across growing season in 2013 (Supplementary Figure 2). Interactions of multiple stresses may lead to a sharp decrease in yield (Mittler, 2006), even when the salt stress is mild. The yield of genotypes tested in the OPTIMISC project was high in European areas without salt stress (Kalinina et al., 2017), but they seemed to be not adapted to the conditions in our field trial, as evidenced by the lowest yields among all accessions without salt stress for the OPM genotypes (below EC 2.5 dS/m). At moderate salt stress (EC 6 dS/m), these genotypes also showed the lowest salt tolerance compared with the Chinese accessions (Figure 3). The accessions collected near the coastal regions (Dongtai, Dongying, and Haizhou) had higher yield than those from the fields in Nanjing where the annual rainfall is much higher than at the location of the field trial (see Figure 3 for yields). The higher amount of rain in Nanjing may reduce chance of soil salinization and drought, which does not favour selection of salt and drought tolerance properties in the prevalent populations in this area. The accessions from Haizhou showed the highest salt tolerance in our study (nearly 70%). For the average yield for each group at EC 6 dS/m, the population from Dongying produced the highest biomass (Figure 3). The high-yield and salt tolerant accessions were mostly collected from coastal regions, suggesting that the accessions growing in these different coastal areas may provide valuable genetic resources to breed salt tolerant *Miscanthus*.

4.2 Useful traits for breeding

High quality for combustion or biogas and biomass yield are two major goals for *Miscanthus* cultivation. Traits related to shoot dry weight are most interesting for anaerobic digestion because the total biomass can be used to produce biogas (Iqbal et al., 2017). The leaf parts are better feedstock than the stem parts due to their easier digestion (van der Weijde et al., 2016a). Leafy genotypes under salt stress may have higher saccharification efficiency in the biogas process, but there is a trade-off between yield and conversion efficiency. The stem/leaf ratio was shown to be decreased by drought stress (van der Weijde et al., 2016b), and a similar trend was observed in our genotype set under salt stress (Table 3): salinity not only decreased the total shoot dry weight but also reduced the stem/leaf ratio in 2014 (Table 3). The accessions CN31, 33, 34, 35, and 36, with stem-to-leaf ratios above 2 at EC 6 dS/m, would represent the best materials for breeding cultivars best suited for combustion purposes. Accession CN31 showed the highest ratio of stem/leaf at high salt stress, which was high even at EC 10 dS/m. This genotype could be used in breeding programs to improve the combustion quality under salt stress through favorable stem/leaf ratios.

Accession CN36 had the highest shoot dry weight and stem dry weight and is one the most promising candidates to produce biomass for both combustion and biogas under moderately saline conditions, and to be included in breeding programs. Under highly saline conditions (EC 10 dS/m), accessions CN14 and CN34 produced relatively high yield with 284 and 302 g/m^2, respectively, and both accession CN14 and CN34 showed higher salt tolerance than the average. Therefore, these two accessions may contribute to the improvement of yield and salt tolerance of the *Miscanthus* crop under high salinity conditions.

Another quality trait that needs to be considered is mineral content. *Miscanthus* genotypes with a lower level of inorganic ions in their harvestable biomass iare particularly important because a high concentration of ions, especially K^+, Na^+, and Cl^-, has a corrosive effect on combustion equipment (Jorgensen, 1997). The concentration of the main minerals in *Miscanthus* varies significantly depending on genotypes, harvest time, location, and fertilization (Lewandowski et al., 2003; Brosse et al., 2012). As salt stress is known to cause accumulation of Na^+ and Cl^-, it is worth to look for *Miscanthus* accessions that have relatively low mineral contents in their harvestable biomass when cultivated under saline conditions. In our field trial, there were no significant differences in Na^+, Ca^{2+}, K^+ and Cl^- contents in both leaves and stems under an EC value of 6 dS/m. This suggests that potential damage to combustion equipment due to corrosion by high mineral contents is unlikely when the *Miscanthus* biomass is produced from fields with an EC value lower than 6 dS/m. Above EC value of 6 dS/m, the Na^+ and Cl^- contents in both leaves and stems were significantly increased, and the K^+ contents significantly increased in stems but not in leaves (Table 4). The Ca^{2+} contents in both leaves and stems did not significantly change with EC values higher than 6 dS/m, while the Mg^{2+} contents in both leaves and stems increased significantly above 4 dS/m (Table 4). The changes of the five ion (Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}) in senesced stems and leaves above EC 6 dS/m agrees with the results of our previous study (Chapter 4, Figure 4), where plants treated with 200 mM NaCl differ from controls. However, a decrease in leaf K^+ contents was observed in young seedlings at 150 mM NaCl for 17 days in a hydroponic system (Chapter 2, Chen et al., 2017) and this was also observed after 6 weeks of 150 mM NaCl treatment in pots at under greenhouses (Chapter 3 Figure 2e). A possible explanation for the difference in response with respect to K^+ may lie in the different development stages of the plants (green leaves vs senesced leaves) and different levels of salt stress. Because high Na^+ concentrations interfere with K^+ uptake and K^+ function, keeping a high K^+ or K^+/Na^+ ratio under salt stress is considered an indicator of salt tolerance (Chen et al., 2007; Shabala and Cuin, 2008). Some *Miscanthus* genotypes using Na^+ exclusion mechanisms to keep low ion contents in the shoot have been identified in a hydroponic system (Chen et al., 2017), and the tested accessions in our field trial appear to be able to maintain low ion contents between EC 2-6 dS/m. As a result, these accessions can produce biomass at mildly saline conditions (below 6 dS/m) that is not compromised for combustion quality by ion accumulation.

4.3 Field management

Although identifying high-yielding and salt tolerant accessions and genotypes for high yield on marginal lands was the main target of this study, it was also interesting to obtain indications of which supplementary cultivation measures may alleviate the consequences of stress and enhance the yields under these marginal conditions. There are several ways to enhance the salt tolerance since salt stress includes osmotic stress, ionic stress, and nutritional imbalance stress (Ashraf and Harris, 2004; Munns and Tester, 2008). The accessions grown in the EC range 2.5-6 dS/m showed a serious reduction but not high ion accumulation compared to that below EC of 2.5 dS/m, indicating the range of EC (2.5-6 dS/m) caused osmotic stress rather than ionic stress. The osmotic stress leads to a greater negative effect on growth rates than the ionic stress (Munns and Tester, 2008), and the biomass indeed had 54 % reduction at EC 6 dS/m in 2014 (Figure 2), suggesting that alleviating osmotic stress may improve the biomass. There was proof of significant interaction between *Miscanthus* genotypes and environmental factors at six contrasting field sites in the studies (Lewandowski et al., 2016; Kalinina et al., 2017). Suitable field management measures should be applied to reduce negative impacts from different climates and locations. For example, in the *Miscanthus* field trial in Adana, Turkey, with a dry and hot climate, extra irrigation was applied to maintain the yield for the first two years (Kalinina et al., 2017). It has been reported that osmotic tolerance in plants could be enhanced by additional N fertilization, which may alleviate osmotic stress by preventing cell membrane damage and improving osmotic regulation (Halvorson and Reule, 1994; Neukirchen et al., 1999; Saneoka et al., 2004). Additionally, N fertilization not only improves biomass yield of *Miscanthus* × *giganteus* by increasing tiller density and weight (Lee et al., 2017) but it also improves root structure, which may increase potential water uptake to overcome conditions of water deficiency (Neukirchen et al., 1999). Due to the competition between Na^+ and K^+, the leaf Na^+ content was higher than leaf K^+ above EC of 6 dS/m (Table 4). Treating with K^+ improved the shoot fresh weight and height in barley under salt stress (Fayez and Bazaid, 2014), suggesting the extra K fertilization may also alleviate ionic stress. Besides applying N and K fertilization, adding specific ions also can improve ion homeostasis under salt stress. For example, calcium and silicon also can improve nutritional and ionic balance to increase the salt tolerance (Yeo et al., 1999; Guimarães et al., 2012; Morgan et al., 2017; Salma Wasti, 2017). Lewandowski et al. (2016) and Kalinina et al. (2017) provided proof of significant interaction between *Miscanthus* genotypes and environmental factors at six contrasting field sites, and suitable field management can reduce negative impacts from both osmotic stress and ionic stress. Therefore, the interaction between field management and responses of different *Miscanthus* genotypes should be studied to further optimize the yield on marginal lands along with genotype selection and improvement of salt tolerance.

5 Funding

The research received funding from the European Union consortium OPTIMISC (project ID 289159). Chang-Lin Chen is supported by a scholarship from the Ministry of Education, Taiwan.

6 Acknowledgments

This work was performed as part of the OPTIMISC project, which received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement No. 289159. The authors gratefully acknowledge expert advice from Oene Dolstra.

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Supplementary material

Supplementary Table 1. The equations (Eqn 1and Eqn 2) for each genotype/accession estimate the dry weight of shoots and stems at the different levels of EC.

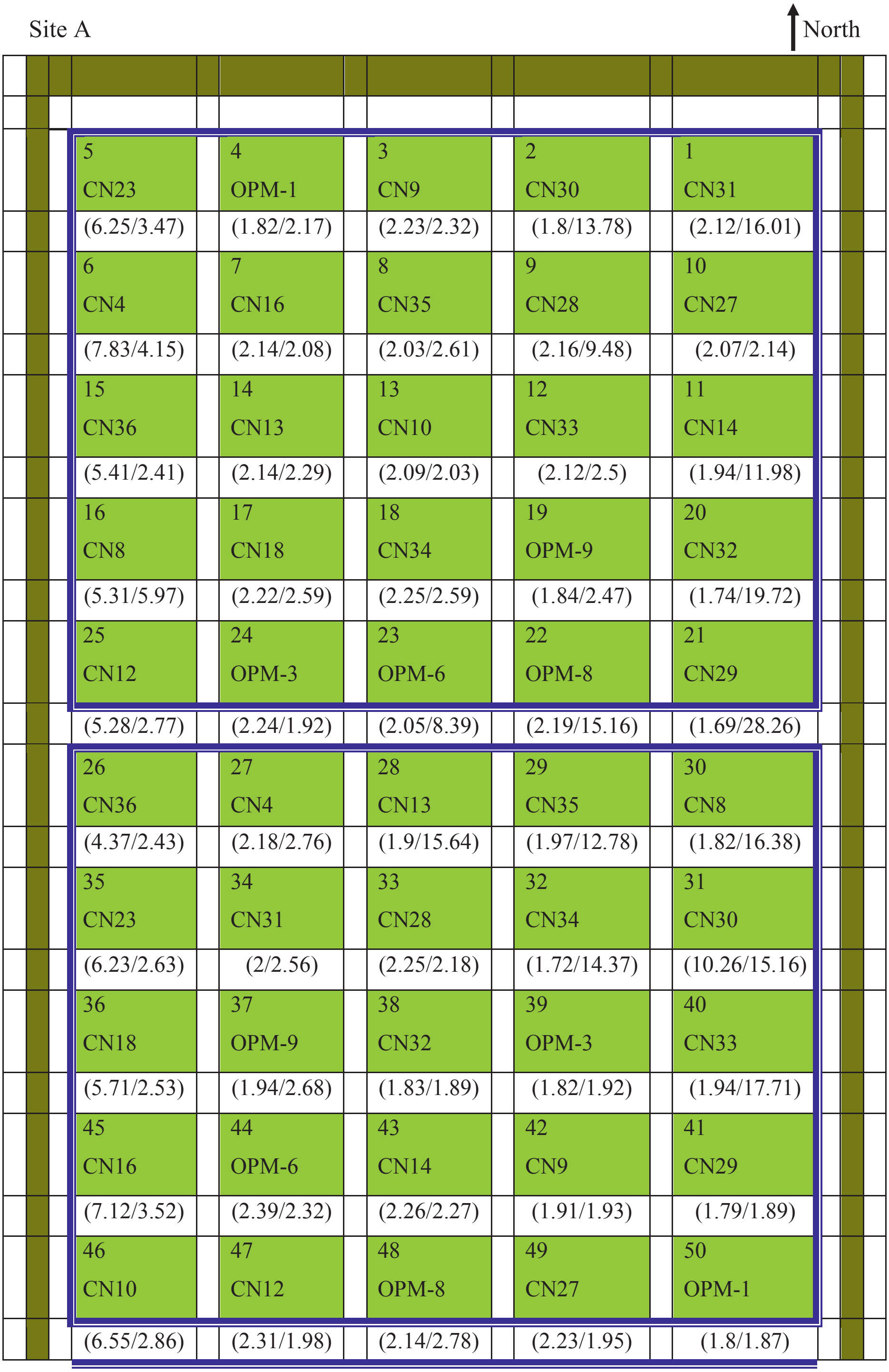
Genotype /Accession	Shoot dry weight				Stem dry weight				Formula
	b_1	b_0	r^2	Sig.	b_1	b_0	r^2	Sig.	
CN4	-0.14	1795.75	0.75	*	-0.17	1390.91	0.76	*	Eqn 1
CN8	-0.17	1711.57	0.84	*	-0.23	1357.16	0.82	*	Eqn 1
CN9	-0.23	1735.35	0.91	**	-0.29	1299.62	0.94	**	Eqn 1
CN10	-0.10	1015.22	0.72	**	-0.13	661.46	0.69	*	Eqn 1
CN12	-0.16	1660.01	0.92	**	-0.19	1057.66	0.94	**	Eqn 1
CN13	-0.14	1572.45	0.86	*	-0.17	934.64	0.89	*	Eqn 1
CN14	-0.06	726.48	0.78	*	-0.09	404.86	0.82	*	Eqn 1
CN16	-0.14	1438.93	0.66	*	-0.18	853.06	0.72	*	Eqn 1
CN18	-0.18	1401.61	0.91	**	-0.25	1097.94	0.94	***	Eqn 1
CN27	-0.08	574.6	0.89	**	-0.10	300.79	0.88	*	Eqn 1
CN28	-214.22	737.73	0.93	**	-131.83	415.64	0.99	***	Eqn 2
CN29	-0.13	521.84	0.91	**	-0.15	207.49	0.83	*	Eqn 1
CN30	-0.29	2029.18	1.00	*	-0.29	1368.55	1.00	*	Eqn 1
CN31	-0.15	886.92	0.95	**	-0.16	676.66	0.95	**	Eqn 1
CN32	-0.27	5284.83	0.94	**	-0.36	6072.74	0.94	**	Eqn 1
CN33	-0.13	1731.2	0.92	**	-0.16	1532.4	0.97	**	Eqn 1
CN34	-0.09	1086.39	0.94	**	-0.10	811.18	0.93	**	Eqn 1
CN35	-561.96	1762.75	0.93	**	-432.99	1325.34	0.92	**	Eqn 2
CN36	-0.13	1956.71	0.79	**	-0.15	1685.81	0.78	*	Eqn 1
OPM-1	-0.33	1800.45	0.95	***	-0.36	1494.76	0.96	***	Eqn 1
OPM-8	-0.12	516.84	0.83	*	-0.13	314.49	0.83	*	Eqn 1

b_0 and b_1 are the constant for different accession/genotypes
*, **, ***, significant at $P < 0.05$; 0.01; 0.001 respectively

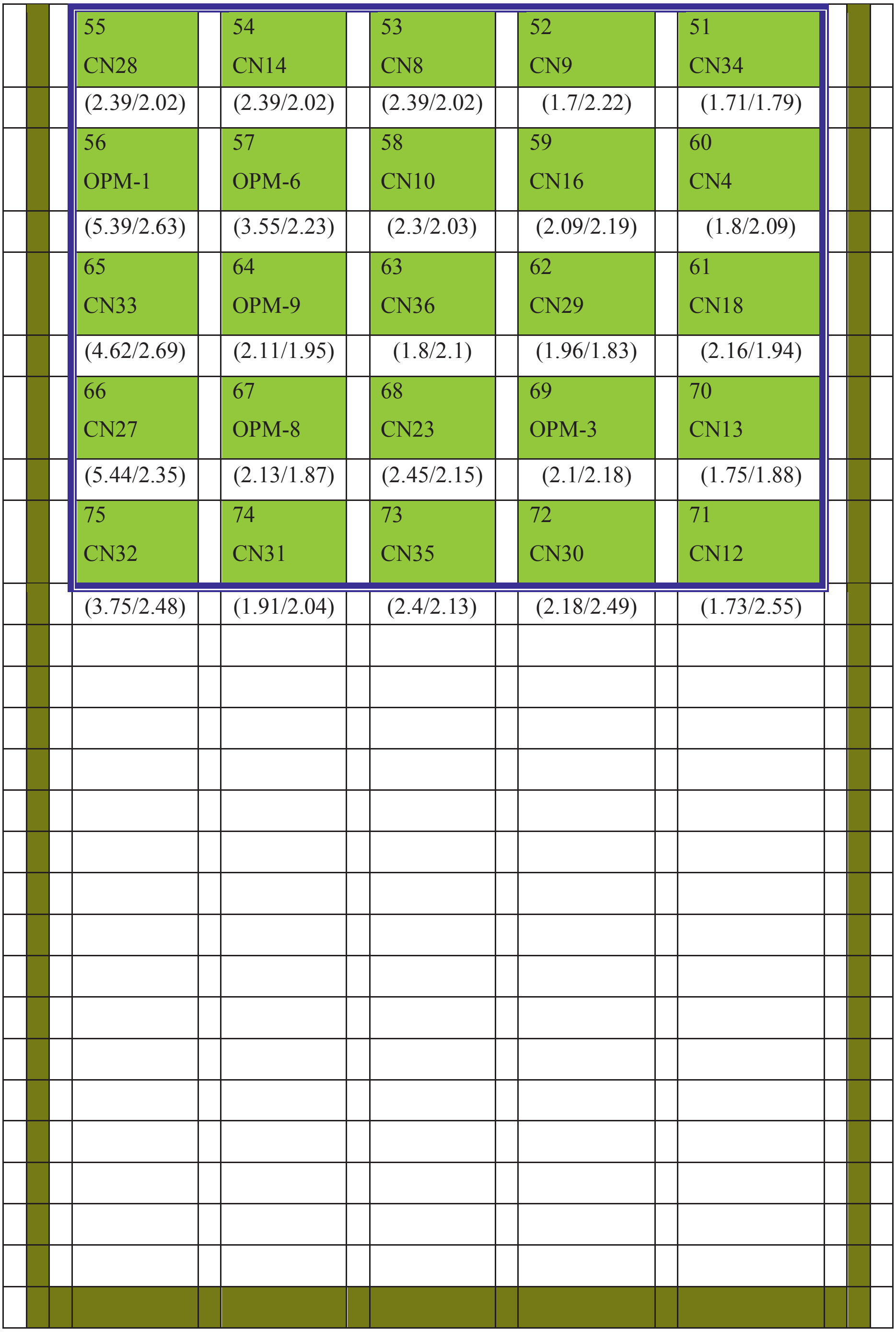
Supplementary Table 2. The nutrient levels of soil in site A and B before planting.

	K_2O (mg/100 g)	P_2O_5 (mg/100 g)	Mg (mg/100 g)
Site A	11.12	3.37	43.07
Site B	14.14	6.78	47.87

Site A

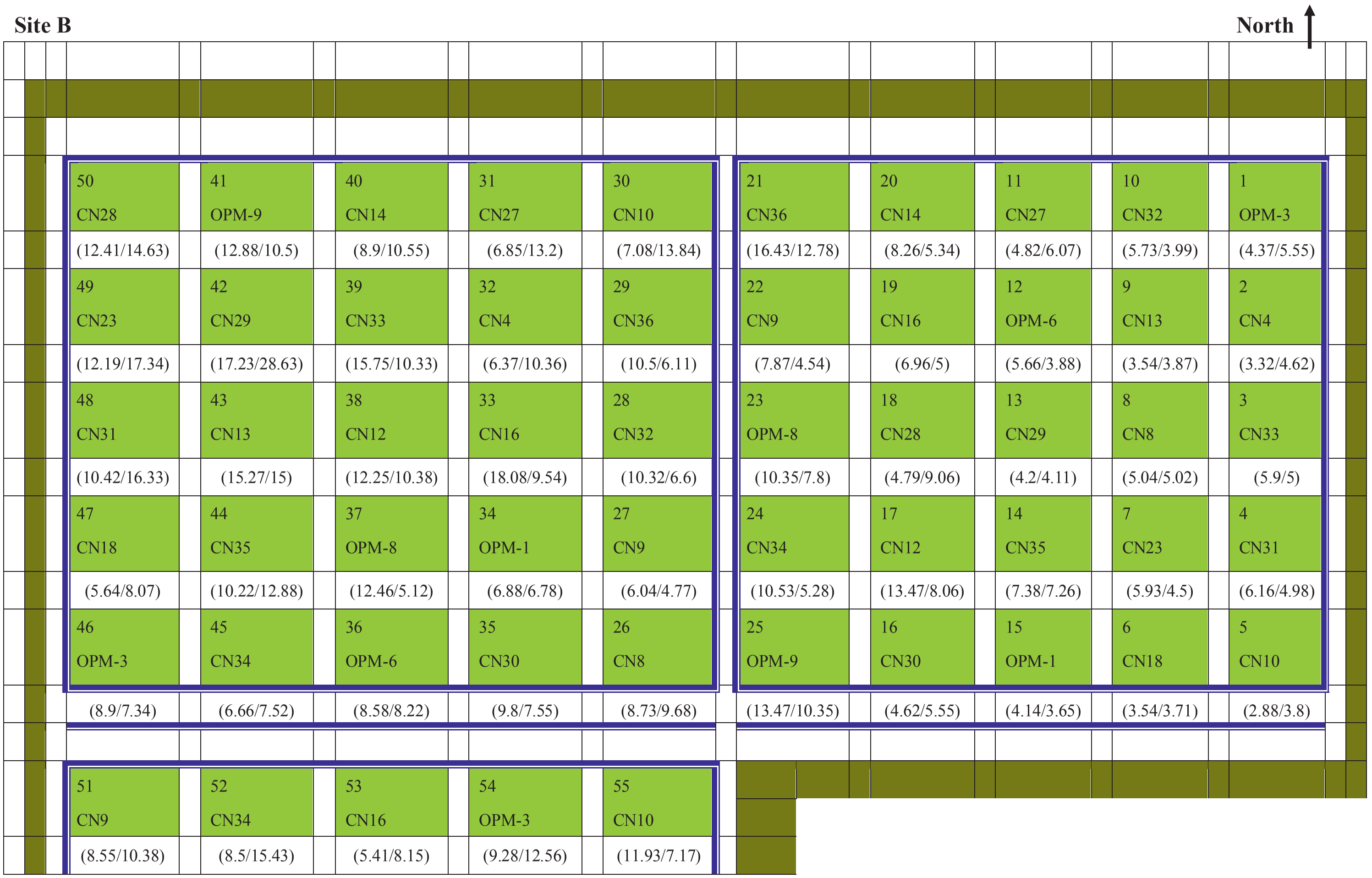


5 CN23 (6.25/3.47)	4 OPM-1 (1.82/2.17)	3 CN9 (2.23/2.32)	2 CN30 (1.8/13.78)	1 CN31 (2.12/16.01)
6 CN4 (7.83/4.15)	7 CN16 (2.14/2.08)	8 CN35 (2.03/2.61)	9 CN28 (2.16/9.48)	10 CN27 (2.07/2.14)
15 CN36 (5.41/2.41)	14 CN13 (2.14/2.29)	13 CN10 (2.09/2.03)	12 CN33 (2.12/2.5)	11 CN14 (1.94/11.98)
16 CN8 (5.31/5.97)	17 CN18 (2.22/2.59)	18 CN34 (2.25/2.59)	19 OPM-9 (1.84/2.47)	20 CN32 (1.74/19.72)
25 CN12 (5.28/2.77)	24 OPM-3 (2.24/1.92)	23 OPM-6 (2.05/8.39)	22 OPM-8 (2.19/15.16)	21 CN29 (1.69/28.26)
26 CN36 (4.37/2.43)	27 CN4 (2.18/2.76)	28 CN13 (1.9/15.64)	29 CN35 (1.97/12.78)	30 CN8 (1.82/16.38)
35 CN23 (6.23/2.63)	34 CN31 (2/2.56)	33 CN28 (2.25/2.18)	32 CN34 (1.72/14.37)	31 CN30 (10.26/15.16)
36 CN18 (5.71/2.53)	37 OPM-9 (1.94/2.68)	38 CN32 (1.83/1.89)	39 OPM-3 (1.82/1.92)	40 CN33 (1.94/17.71)
45 CN16 (7.12/3.52)	44 OPM-6 (2.39/2.32)	43 CN14 (2.26/2.27)	42 CN9 (1.91/1.93)	41 CN29 (1.79/1.89)
46 CN10 (6.55/2.86)	47 CN12 (2.31/1.98)	48 OPM-8 (2.14/2.78)	49 CN27 (2.23/1.95)	50 OPM-1 (1.8/1.87)

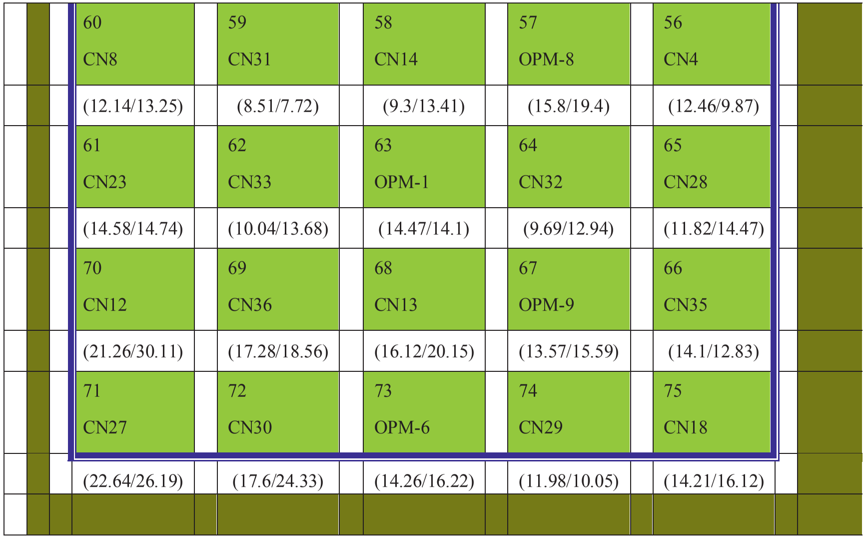


55 CN28	54 CN14	53 CN8	52 CN9	51 CN34
(2.39/2.02)	(2.39/2.02)	(2.39/2.02)	(1.7/2.22)	(1.71/1.79)
56 OPM-1	57 OPM-6	58 CN10	59 CN16	60 CN4
(5.39/2.63)	(3.55/2.23)	(2.3/2.03)	(2.09/2.19)	(1.8/2.09)
65 CN33	64 OPM-9	63 CN36	62 CN29	61 CN18
(4.62/2.69)	(2.11/1.95)	(1.8/2.1)	(1.96/1.83)	(2.16/1.94)
66 CN27	67 OPM-8	68 CN23	69 OPM-3	70 CN13
(5.44/2.35)	(2.13/1.87)	(2.45/2.15)	(2.1/2.18)	(1.75/1.88)
75 CN32	74 CN31	73 CN35	72 CN30	71 CN12
(3.75/2.48)	(1.91/2.04)	(2.4/2.13)	(2.18/2.49)	(1.73/2.55)

Site B

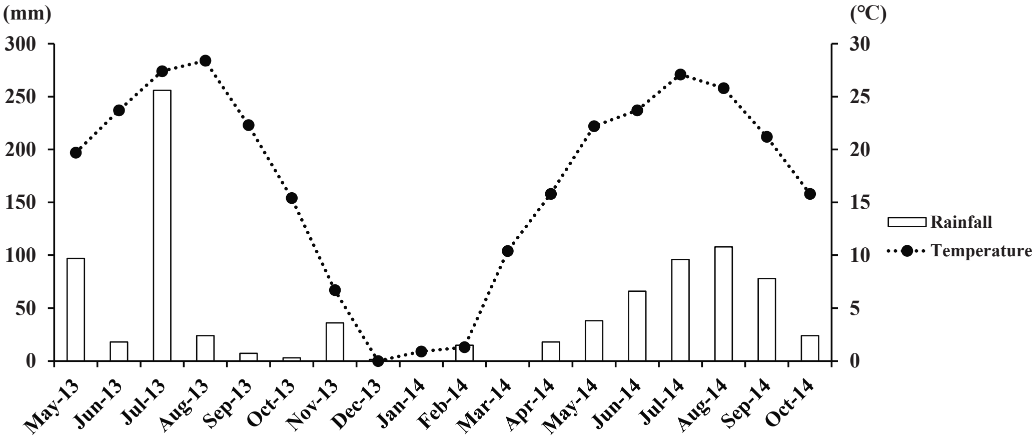


50 CN28 (12.41/14.63)	41 OPM-9 (12.88/10.5)	40 CN14 (8.9/10.55)	31 CN27 (6.85/13.2)	30 CN10 (7.08/13.84)	21 CN36 (16.43/12.78)	20 CN14 (8.26/5.34)	11 CN27 (4.82/6.07)	10 CN32 (5.73/3.99)	1 OPM-3 (4.37/5.55)
49 CN23 (12.19/17.34)	42 CN29 (17.23/28.63)	39 CN33 (15.75/10.33)	32 CN4 (6.37/10.36)	29 CN36 (10.5/6.11)	22 CN9 (7.87/4.54)	19 CN16 (6.96/5)	12 OPM-6 (5.66/3.88)	9 CN13 (3.54/3.87)	2 CN4 (3.32/4.62)
48 CN31 (10.42/16.33)	43 CN13 (15.27/15)	38 CN12 (12.25/10.38)	33 CN16 (18.08/9.54)	28 CN32 (10.32/6.6)	23 OPM-8 (10.35/7.8)	18 CN28 (4.79/9.06)	13 CN29 (4.2/4.11)	8 CN8 (5.04/5.02)	3 CN33 (5.9/5)
47 CN18 (5.64/8.07)	44 CN35 (10.22/12.88)	37 OPM-8 (12.46/5.12)	34 OPM-1 (6.88/6.78)	27 CN9 (6.04/4.77)	24 CN34 (10.53/5.28)	17 CN12 (13.47/8.06)	14 CN35 (7.38/7.26)	7 CN23 (5.93/4.5)	4 CN31 (6.16/4.98)
46 OPM-3 (8.9/7.34)	45 CN34 (6.66/7.52)	36 OPM-6 (8.58/8.22)	35 CN30 (9.8/7.55)	26 CN8 (8.73/9.68)	25 OPM-9 (13.47/10.35)	16 CN30 (4.62/5.55)	15 OPM-1 (4.14/3.65)	6 CN18 (3.54/3.71)	5 CN10 (2.88/3.8)
51 CN9 (8.55/10.38)	52 CN34 (8.5/15.43)	53 CN16 (5.41/8.15)	54 OPM-3 (9.28/12.56)	55 CN10 (11.93/7.17)					

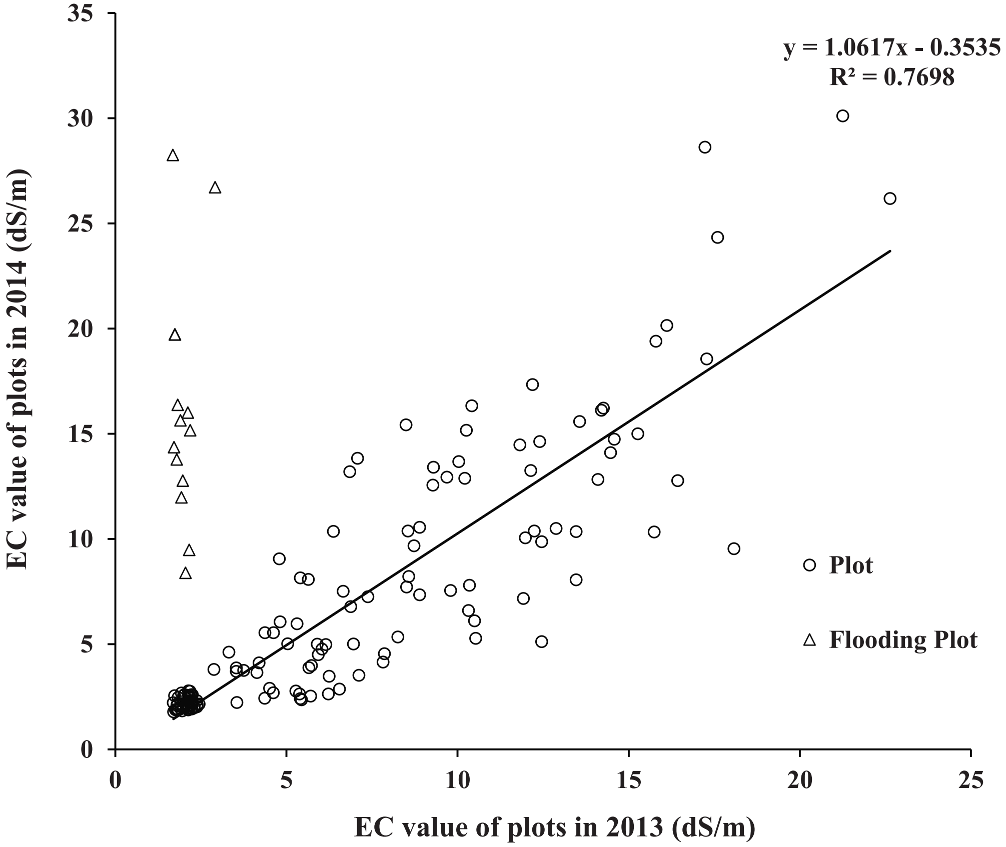


60 CN8	59 CN31	58 CN14	57 OPM-8	56 CN4
(12.14/13.25)	(8.51/7.72)	(9.3/13.41)	(15.8/19.4)	(12.46/9.87)
61 CN23	62 CN33	63 OPM-1	64 CN32	65 CN28
(14.58/14.74)	(10.04/13.68)	(14.47/14.1)	(9.69/12.94)	(11.82/14.47)
70 CN12	69 CN36	68 CN13	67 OPM-9	66 CN35
(21.26/30.11)	(17.28/18.56)	(16.12/20.15)	(13.57/15.59)	(14.1/12.83)
71 CN27	72 CN30	73 OPM-6	74 CN29	75 CN18
(22.64/26.19)	(17.6/24.33)	(14.26/16.22)	(11.98/10.05)	(14.21/16.12)

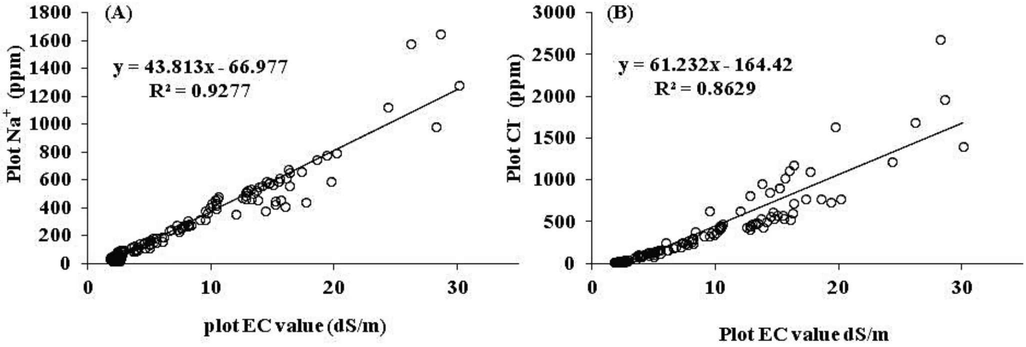
Supplementary Figure 1. Field plans of random block were shown. Each plot occupied 4 square meters and the one-meter row located between two plots. EC value is presented in the parenthesis (EC value of 2013 /EC value of 2014).



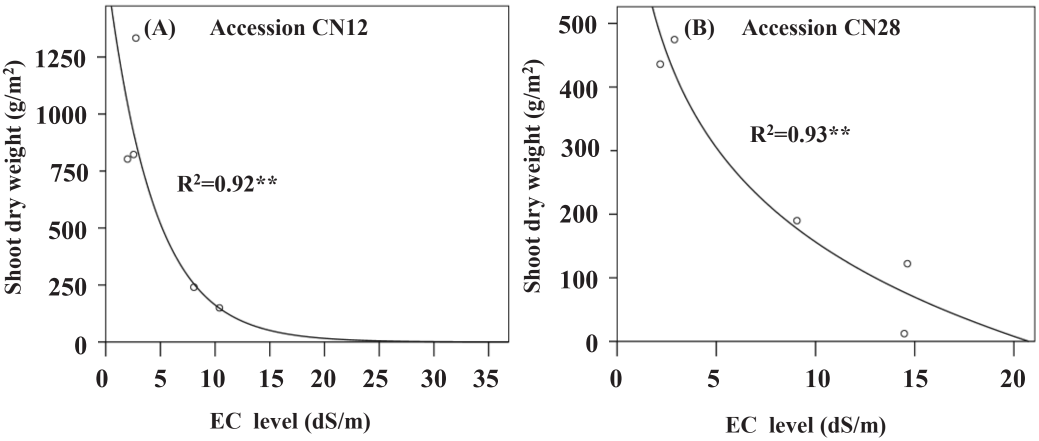
Supplementary Figure 2. Mean air temperature and rainfall during two growing seasons (from Jun 2013 until Oct 2014).



Supplementary Figure 3. The relationship of plot EC value between 2013 and 2014. The flooding plots (triangle) with sharp changes between 2013 and 2014 were taken out before analysis.



Supplementary Figure 4. There was strong relationship between plot EC value and Na^+ and Cl^- of the solution (1:2 soil to water extract) in 2014.



Supplementary Figure 5. The changes in shoot dry weight with EC value in 2014 fit the curve of the equation 1 for accession CN12 (A) and the equation 2 for accession CN28.

Chapter 6

General discussion

Miscanthus is a perennial grass with a C4 photosynthetic pathway and high biomass production and resource-use efficiency, which makes it favorable for bioenergy production (Zub and Brancourt-Hulmel, 2010). *Miscanthus* is utilized mostly for producing heat by direct combustion or for generating biofuel such as biomethane or bioethanol by anaerobic digestion. The two different purposes set different demands on biomass quality. Avoiding low melting temperatures and corrosive issues for combustion requires low mineral contents in the harvested biomass (Zub et al., 2011). Enhancing conversion efficiency from biomass to biogas needs digestable cell wall properties, with cell walls consisting of high hemicellulose but low lignin, which is considered the better quality for biofuel production (van der Weijde et al., 2016a; van der Weijde et al., 2017a; van der Weijde et al., 2017b).

Miscanthus is expected to be grown on marginal lands that are exposed to abiotic stresses such as salinity, to avoid competition with food crops on agricultural lands (Lewandowski et al., 2016). Soil salinity decreases crop yield and may also affect quality of the biomass, so identifying salt tolerant genotypes for cultivation on saline soils, and genotypes that have traits that can be used for improvement of salinity tolerance are important goals to be able to extend cultivation to marginal lands. Although salt tolerance, yield, biomass quality and ion contents are important traits for *Miscanthus*, these traits may be challenging for breeders because of genetic complexity and interaction with environmental conditions. Breeding for salt tolerant plants is certainly not straightforward. Some of these challenges can be met by performing evaluations under different conditions. In this thesis, we used different screening environments, including a hydroponics system, pot experiments in (semi) protected environments and field conditions, to evaluate genetic variation for salt tolerance, identify mechanisms that confer salt tolerance, and identify salt tolerant *Miscanthus* genotypes and traits contributing to salt tolerance.

First, a large screen was done with young vegetative plants in a hydroponics system. In Chapter 2, we evaluated genetic diversity for salt tolerance in a set of 70 genotypes and identified suitable traits, such as low shoot Na^+ ion contents, that contribute to salt tolerance and can be used for improving salt tolerance in breeding programs. Based on the results in Chapter 2, twelve genotypes with interesting salt tolerance properties were selected for pot experiments with different salt stress levels (0, 150, and 250 mM NaCl) in Chapter 3. The low Na^+ ion contents in shoots and relatively high Na^+ ion contents in roots at 150 mM NaCl indicated that all selected genotypes use Na^+ exclusion mechanisms at mild salinity level, while the high Na^+ ion accumulation in shoots of most genotypes at 250 mM NaCl suggested that most genotypes did not have the ability to still effectively exclude Na^+ from the shoots at higher salt stress levels. Furthermore, the results indicate that the rhizome may interact with salt tolerance traits, which has implications for the choice of planting material, and may suggest that rhizome properties should be included in improving salt tolerance of *Miscanthus*. We further explored the effect of salinity on quality of the different types of biomass in Chapter 4. Twelve genotypes were tested at 150 mM NaCl stress for 6 weeks (green biomass) and six genotypes were exposed to 200 mM NaCl and control conditions for 19 weeks (senesced biomass). Analyses of cell wall composition in the stems at 150 mM NaCl for 6 weeks showed an increase in hemicellulose content and a reduction in cellulose. This change in cell wall composition is likely to positively affect saccharification efficiency (van der Weijde et al., 2016b). However, when *Miscanthus* was exposed to 200 mM NaCl for 19 weeks, there was an increase in lignin and a reduction in hemicelluloses compared with control conditions, and high lignin content is not beneficial for saccharification efficiency (van der Weijde et al., 2016a). The results in this chapter show that salinity affects cell wall composition in such a way that it may influence its suitability for bioenergy production, but that the effects are dependent on harvest time (type of harvest), and severity of the salinity. As *Miscanthus* is a perennial field crop, we evaluated the performance of over 20 genotypes/accessions, including a number of accessions collected in China, in field trials in China for two years in Chapter 5. This chapter illustrates the challenges of doing reproducible field trials for salt tolerance evaluations with highly heterogeneous conditions, which were partly counteracted by measuring soil properties at the plot level. Although there was little overlap in genotypes tested in the field trials and in the other chapters under controlled conditions (hydroponics system and pot experiments), it is clear that the Chinese accessions are a valuable addition to *Miscanthus* germplasm for traits that contribute to growth under marginal saline conditions. The results in this thesis thus provide interesting information on how to use the different screening systems to identify stable and potentially salt tolerant genotypes with high quality and quantity biomass at acceptable salt levels.

1 Screening systems for salt tolerance: hydroponics vs pots vs field

Soil salinity decreases crop yield and quality so identifying salt tolerant genotypes for improvement of salinity tolerance is an important target. However, breeding for salt tolerance in plants is not straightforward due to its genetic complexity and interaction with the environment (Flowers and Flowers, 2005). Tavakkoli et al. (2012) used a hydroponic system to identify potential genotypes in 60 barley genotypes and 15 genotypes with different salt tolerance and ion exclusion efficiency were tested in pots and fields. They found significant differences in performance and ion accumulation between pots and hydroponics. We used a similar strategy to identify genetic variation of salt tolerance and mechanisms that confer salt tolerance and to validate identified salt tolerant genotypes and traits contributing to salt tolerance by using different screening systems including a hydroponics system and pots, as well as field conditions.

1.1 Hydroponics

Hydroponic growth systems not only have a high capacity for a large number of genotypes at the same time but also supply uniform conditions for the root environment (Munns and James, 2003; Almeida et al., 2016). Hydroponic systems, with a highly controlled environment, enable the selection of genetic traits contributing to salt tolerance with little or no environmental variation masking the genetic variation. Using such a system, high potential genotypes for breeding purposes can be identified that have interesting salt tolerance traits, using phenotyping methods such as ion content and leaf expansion measurements. Indeed, several studies on salt tolerance using hydroponic systems found correlations between salt tolerance and Na^+ and K^+ concentrations in shoots and between osmotic tolerance and reduction of the leaf area (Munns and James, 2003; De Costa et al., 2007; Jaarsma et al., 2013; Platten et al., 2013). In our hydroponics screen described in Chapter 2, ion contents (including Na^+, Cl^-, and K^+) were measured and correlated to physiological and growth traits. A positive relationship between leaf K^+/Na^+ ratio and shoot dry weight and a negative correlation between leaf K^+/Na^+ ratio and senescence at 150 mM NaCl salt stress were found. In addition, the leaf Cl^- and Na^+ concentrations were negatively correlated to the shoot biomass (r =-0.43 and -0.53, respectively) and postively correlated to senescence (r =0.41 and 0.54, respectively). These results indicate that maintaining low leaf Na^+ and Cl^- concentrations and high K^+/Na^+ ratio under salt stress are important traits for *Miscanthus* growth under salt stress, at least under hydroponic conditions. Based on the ion content data, six genotypes (OPM-4, 32, 37, 59, 69, and 71) with a high K^+/Na^+ ratio (twice higher than the average value) may be utilizing a shoot Na^+-exclusion mechanism under saline conditions (Chapter 2, Table 4). It is interesting that genotype OPM-31 showed the highest salt tolerance across 70 genotypes in our hydroponic system, and also showed the highest drought tolerance across 49 genotypes in pot experiments (van der Weijde et al., 2016b). This genotype may use osmotic tolerance mechanisms to increase salt tolerance rather than Na^+ exclusion mechanisms because the leaf K^+/Na^+ ratio was only just above the average (Chapter 2). Osmotic stress typically leads to a greater reduction of growth rates than ionic stress (Munns and Tester, 2008). Genotype OPM-67 had the eight-highest salt tolerance of 70 genotypes and ranked 6th out of 49 genotypes for drought tolerance (van der Weijde et al., 2016b). Interestingly, genotype OPM-67 even had the second lowest K^+/Na^+ ratio in shoots. This genotype may also utilize osmotic mechanisms rather than ionic exclusion mechanisms to deal with salt stress. Alternatively, it may be highly effective in compartmentalizing ions in the vacuole, using a tissue tolerance mechanism. The commercial genotype OPM-9 (*Miscanthus* × *giganteus*) had 42% salt tolerance at 150 mM NaCl which was close to the average of 70 tested genotypes (43%). Our study indicates that there are several genotypes with high salt tolerance and yield under salt stress which surpass *Miscanthus* × *giganteus* under hydroponic conditions and thus may be a valuable resource for breeding and production.

1.2 Hydroponics vs pot experiments

In order to explore the applicability of the hydroponics selection for genotypes in *Miscanthus*, we addressed the question: Would these traits in young seedlings under hydroponics system also be expressed and contribute to salt tolerance when the plants are grown in a more realistic environment for the roots, like in pots in the greenhouse? It is important to realize that perennials like *Miscanthus* grow for a prolonged period and have rhizomes that help them to survive under harsh conditions such as in winter. Moreover, it is not unlikely that soil texture and composition may influence salinity tolerance and root properties related to soil traits, and these may have a different impact on growth and yield (Tavakkoli et al., 2012). In addition, plants extensively interact with the soil microbiome, and these interactions can be highly beneficial in particular under stress conditions (Qin et al., 2016). Although root traits can be easily accessed in a hydroponic system, the root behavior and the consequences of genetic variation in root traits for growth may therefore differ significantly from plants grown in soil. The influence on salt tolerance of rhizomes in perennials like *Miscanthus* may be considerable, and this cannot be properly evaluated in a hydroponics system. In order to better understand the role of rhizomes in salt tolerance, two sets of plants were compared in pots in Chapter 3: plants started from rhizomes collected from field experiments, and plants started from *in vitro* culture, subsequently propagated on a hydroponic system. We did not use potting soil but vermiculite, to avoid salt accumulation over time in the pots. The rhizome-started plants were at the 7-leaf stage and *in vitro*-started plants were at the 10-leaf stage at the start of the pot study, compared to young seedlings (4-leaf stage) in the hydroponics system. In this pot experiment, 7 of the 12 genotypes with rhizomes demonstrated a significant reduction in senescence at 250 mM NaCl compared with *in vitro*-started plants. The rhizome-based plants had less senescence than the *in* vitro-started plants, but this was not linked to lower ion contents, which suggests that rhizomes may enhance the osmotic tolerance under salt stress or are somehow able to counteract the effects of high ion content on senescence. Two genotypes (OPM-31 and 67) showed no significant differences in senescence between rhizome-based plants and *in vitro*-plants at 0, 150, and 250 mM NaCl (Chapter 3, Table 2), suggesting both genotypes may have the ability to delay senescence independent of the rhizome, possibly through better management of Reactive Oxygen Species (ROS) accumulation, or osmotic adjustment (Jajic et al., 2015). This would agree with OPM-31 and 67 being the more drought tolerant genotypes of 49 tested genotypes (van der Weijde et al., 2016b).

There were no significant differences in leaf Na^+ between 0 and 150 mM NaCl in the pot experiments in Chapter 3, suggesting that the salt stress applied was not high enough for Na^+ to accumulate in the leaves. However, the 150 mM NaCl stress in the hydroponic system did cause ion accumulation in shoots, even within 3 weeks. This discrepancy may be caused by the fact that the plants in the pot experiments were older than that in the hydroponics system. Alternatively, the continuous exposure to salt and the liquid root environment (with higher diffusion rates) may contribute to the stronger salt effect and ion uptake in the hydroponics experiments. At more severe salinity (250 mM NaCl), the 12 tested genotypes showed genotypic variation in the leaf Na^+ contents. There was also a positive correlation between senescence and leaf Cl^- (r =0.8) and Na^+ (r =0.79) while senescence negatively correlated with leaf K^+/Na^+ (r =-0.71) of *in vitro*-started plants (bot not in rhizome-derived plants) at this salt stress level in pots. This correlation was also observed at 150 mM NaCl in a hydroponics system, suggesting that the 250 mM NaCl conditions in pots imposed a stress that may be more similar to the stress imposed by 150 mM NaCl in the hydroponic system. However, the relative differences in ion contents between the genotypes were not correlated between these experiments. Three genotypes (OPM-42, 48, and 56) showed low ion accumulation in shoots both in hydroponics system (150 mM NaCl) and pot conditions (250 mM NaCl), but genotype OPM-9 and 71 had low leaf Na^+ ion accumulation in the hydroponic system at 150 mM NaCl but high leaf Na^+ ion contents in pot conditions (250 mM NaCl), while genotype OPM-67 had high leaf Na^+ ion accumulation in hydroponic system (150 mM NaCl) but low leaf Na^+ ion contents in pot conditions (250 mM NaCl). Their opposite performances in different screening systems may not only be due to the different systems, hydroponics vs pots, but may also be related to differences in growth stage, time exposed to the stress, and different salt levels.

1.3 Hydroponics vs pot experiments vs field experiments

For a perennial such as *Miscanthus* productivity ideally needs to be monitored for several years because it reaches its full yield potential 2 to 3 years after planting (Clifton-Brown et al., 2017). In our field trial presented in Chapter 5, the second-year yield was indeed much higher than the first-year yield, and the reduction of yield by salinity in the second year was less than the first year (Chapter 5, Figure 2). Moreover, the root environment is quite different under field conditions, particularly when compared to the hydroponics system, and different types of soil also influence the plant's response to salt stress. This can only be tested in field trials. We have shown that the rhizome can interact with salt tolerance in Chapter 3, with sometimes contrasting responses between rhizome-derived and *in vitro*-started material. Plazek et al. (2014) showed that *Miscanthus* × *giganteus* (OPM-9) with bigger rhizomes were more tolerant. The size of rhizomes can vary between genotypes; the rhizome dry weight of *Miscanthus* × *giganteus* for instance is double that of *M. sinensis* under normal conditions (Christensen et al., 2016). The development and growth of rhizomes under salt stress over a longer period and several seasons is still largely unknown. If long-term salt stress affects the size and development of rhizomes, these variations in rhizome traits may also influence growth under salt stress. The reasons for different behavior between field trials and other evaluation systems thus include the rhizome effects, root development, soil texture and environmental factors, and developmental/phenological stages of the tested plants.

The ion contents in leaves and stems of plants grown in pots at 200 mM for 19 weeks (Chapter 4) and those of the plants in the field trial, harvested when these started to senesce at an EC value of 6 dS/m in the second year (Chapter 5), showed a similar trend: Leaf Na^+, Cl^-, and Mg^{2+} increased and leaf K^+ and Ca^{2+} were similar under salt stress compared with control conditions. Na^+, Cl^-, K^+ and Mg^{2+} concentrations increased in the stems, and Ca^{2+} contents still did not change under salt stress. Although the trends were similar, the above-ground tissue Na^+ and Cl^- ion contents were much higher in plants grown in the pots than in the field. The estimated salt stress level in the field was lower (EC of 6 dS/m is mild stress, comparable to 60-70 mM NaCl), which may account for the difference in ion accumulation. Yet, the biomass of the field-grown genotypes was reduced by 54%. This may be related to the marginal conditions under which the field trial was performed, with no fertilization and limited water management. The field trial site at Dongying used for our study was therefore most likely poor in nutrients, as opposed to the pot experiments under controlled conditions that used a 0.5X Hoagland solution with different salt concentrations. Plants may be more seriously damaged due to a combination of different stresses in the marginal field site at Dongying compared with controlled conditions in greenhouses, i.e. drought stress and nutrient limitation in addition to the salt stress. With sufficient nitrogen available, nitrate may not only improve osmotic tolerance but also reduce the Cl^- accumulation under salt stress (Kafkafi et al., 1982; Fayez and Bazaid, 2014). The interaction between the environmental factors and the response to salt stress of the genotypes should be explored in the future. Although field trials are most relevant for the practical cultivation conditions, the changing and varying conditions in the field compromise reliability of the results and of selections, and this is one of the main complications for screening perennials for stress tolerance under field conditions. It will be interesting to also test the genotypes/accessions that were evaluated in the field trial in the hydroponic system and in pots, as well as under more stable conditions in the field to further increase our insight on the relationships between different screening systems with respect to the performance of *Miscanthus* genotypes under salt stress.

2 Tolerance mechanisms for salt stress

In order to deal with both osmotic and ionic stress, plants undergo a variety of adaptations at the cellular, tissue and whole plant level that help them to grow successfully under salt stress. Salt tolerance is a complex process and multiple genes are involved the physiological and metabolic regulation which reduces the evaporation, keeps the turgor balance and maintains ion homeostasis in plants under salt stress. Measuring a variety of traits, such as stomatal conductance, leaf expansion rate, osmotic adjustment, and ion contents under salt stress helps to understand which mechanisms underlie salt stress tolerance.

2.1 Osmotic tolerance

Osmotic regulation helps plants to minimize the negative effects of salinity-induced osmotic stress. Wheat leaf expansion is considered as a indicator, with nondestructive measuring, for osmotic tolerance (Farouk, 2011). In our tested 70 *Miscanthus* genotypes, there was a highly significant correlation (r =0.86) between leaf expansion rate and shoot dry weight at 150 mM NaCl (Chapter 2). Genotypes OPM 31 and OPM-67 were salt tolerant, and also had high drought tolerance (van der Weijde et al., 2016b), possibly indicating that they utilize osmotic tolerance mechanisms to increase salt tolerance as well as drought tolerance. Others have shown that the proline content in leaves of *Miscanthus* × *giganteus* increased two fold at EC 7.96 dS/m, 48-fold at EC 10.65 dS/m and 142-fold at EC 17.5 dS/m NaCl compared with control conditions (Stavridou et al., 2016). Likewise, it was 6-fold at 100 mM NaCl and 17-fold at 150 mM NaCl relative to the control level (Plazek et al., 2014). Although the proline content in roots was not measured in these experiments, salt stress enhances proline accumulation in barley roots (Ueda et al., 2007) and osmotic stress increases proline accumulation in maize roots (Verslues and Sharp, 1999). Proline is synthesized in the shoot and root under osmotic stress, but it can also be transported via the phloem to the root by proline porters such as ProT2 in white clover (Bok-Rye et al., 2009; Kavi and Sreenivasulu, 2014). We also observed that the presence of rhizomes may delay senescence, a symptom that may also imply enhanced osmotic tolerance (Chapter 3). Rhizomes may support osmotic adjustment or production of compatible solutes such as sugar and amino acids because rhizomes are storage organs that can store nutrients and starch, possibly facilitating production of compatible solutes such as proline and glycine betaine for osmotic adjustment or osmotic protection, enhancing osmotic tolerance (Ings et al., 2013). Therefore, it would be worthwhile to study genetic variation in compatible solute production between high and low osmotic stress tolerant genotypes.

2.2 Ion homeostasis

2.2.1 Na^+ homeostasis

For glycophytes, an exclusion mechanism for limiting transport of sodium ions from the roots to the shoots is an important adaptive trait for plants under salt stress, which helps plants to avoid toxic ion levels in photosynthetic tissues that are important for growth. The average Na^+ root/shoot ratio was around 3 across 70 *Miscanthus* genotypes at 150 mM NaCl in the hydroponics system (Chapter 2) and 6 across 12 *Miscanthus* genotypes in pot experiments at 250 mM NaCl (Chapter 3). These Na^+ ratios of root/shoot (3-6) indicate that the *Miscanthus* genotypes actively avoided Na^+ accumulation in the shoots. Until now, no Na^+-transport related genes or QTLs related to Na^+ exclusion have been reported for *Miscanthus*. However, important genes which were shown to be able to restrict Na^+ accumulation in the shoots were found in other members of the Poaceae family, such as *SCK1* (*HKT1;5*) in rice (Lin et al., 2004), *Nax1* and *Nax2* in durum wheat (Huang et al., 2006; Byrt et al., 2007), *ZmHKT1* in maize (Zhilei et al., 2015). HKT1;5 as a transporter takes Na^+ from the xylem into the parenchyma cells to minimize the accumulation of Na^+ in the shoots. Seven major and three minor alleles of *OsHKT1;5* were identified in rice germplasm, and there was high correlation between the leaf Na^+ concentrations and *HKT1;5* allelic variation across diverse accessions (Platten et al., 2013). Similarly, maize *ZmHKT1* of 66 tested inbred lines was classified into 12 haplotypes and difference of expression levels in inbred lines were detected under salt stress (Zhilei et al., 2015). The *ZmHKT1* expression level was significantly up-regulated in salt tolerant inbred lines under salt stress (Zhilei et al., 2015). Based on this information, it is reasonable to assume that a *HKT1;5* like gene may play a role in Na^+ exclusion in *Miscanthus*. Therefore, in addition to the experiments presented in the previous chapters of this thesis, we used a PCR strategy to isolate *HKT1;5*-like genes from *Miscanthus.* Around 2 kb length of DNA fragments were isolated and sequenced. The fragments showed high similarity with HKT genes in sorghum and maize (92 % and 88%, respectively) and we found 4 alleles in 6 *Miscanthus* genotypes (Figure 1). Additionally, we measured RNA expression levels by real-time PCR for this gene in selected *Miscanthus* genotypes grown at three salt levels (0, 150, and 250 mM NaCl) in a hydroponic system for 12 days. Plants were harvested after 7-day treatments and 12-day treatment to compare the expression levels. The results did not indicate that this gene was up-regulated under salt stress (not shown), suggesting that contrary to expectations, HKT1;5 may not play an important role in Na^+ shoot exclusion in *Miscanthus*.

The NHX gene family also regulates Na^+ homeostasis to improve salt tolerance, but does so by mediating the sequestration of Na^+ into the vacuole under salt stress (Bassil et al., 2012). For example, the root cells of maize accumulate Na^+ in vacuoles through NHX transporters to minimize sodium transport to shoots and lower the osmotic potential under salt stress (Zörb et al., 2005). Since *Miscanthus* and maize are both C4 plants and members of the Poaceae family, *Miscanthus* may use a similar Na^+ exclusion mechanism in response to salt stress. It would be interesting to examine allelic variation and expression of NHX genes under saline conditions in salt tolerant and salt sensitive *Miscanthus* genotypes, and possibly extend these expression studies to the transcriptome to find out which genes may be responsible for regulation of the Na^+ concentrations under salt stress.

2.2.2 K^+ homeostasis

Under salt stress, high Na^+ concentrations interfere with K^+ uptake and K^+ function (Shabala and Cuin, 2008). Maintaining a high K^+ concentration under salt stress is another important mechanism and the K^+/Na^+ ratio is considered as an indicator of salt tolerance (Munns and James, 2003). Almost 90% of 69 barley cultivars had an active K^+ maintenance mechanism to retain cytosolic K^+ concentrations, but only genotypes with high K^+/Na^+ ratio showed high tolerance because they also had relatively low Na^+ contents (Chen et al., 2007). Similarly, salt tolerant maize genotypes maintained a relatively high K^+/Na^+ ratio and produced more biomass than salt sensitive ones under salt stress conditions (Akram et al., 2010). The average leaf Na^+ concentrations of the 70 tested *Miscanthus* genotypes in Chapter 2 increased significantly but leaf K^+ concentrations significantly decreased at 150 mM NaCl compared with control conditions in hydroponic system. Our pot experiments however indicated that regulation of leaf K^+ concentrations is salt stress level-dependent: Both leaf Na^+ and K^+ concentrations significantly increased at 250 mM NaCl compared with 150 mM NaCl. The average K^+/Na^+ ratio of the 70 tested genotypes at 150 mM NaCl in the hydroponic system was not only positively correlated (r =0.56) to shoot dry weight, but also negatively correlated (r =-0.59) to senescence under salt stress (Chapter 2). Similarly, the leaf K^+/Na^+ ratio of the 12 *in vitro*-started plants at 250 mM NaCl in pots was negatively correlated (r =-0.71) to senescence, but not in the rhizome-based plants. This ratio in rhizome-based plants did show a highly positive correlation with shoot dry weight. Thus, maintaining high K^+/Na^+ ratio appears to be an important mechanism to enhance *Miscanthus* salt tolerance at 250 mM NaCl, but the rhizome seems to interact with the consequences of variation in K^+/Na^+ ratios.

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Allel-1         ATGCCCCCTTTGCACGTCCCTAGCAGTAGTAGTGCTCGCAAGTTTTACCACTTTGTGCTCTTCCATGTGCACCCGTTCTGGGTCCACCTCTTGTACTTCCTTTCCATCTCCGTCTTGGGT 120
Allel-2         ATGCCCCCTTTGCACGTCCCTAGCAGTAGTAGTCCTCGCAAGTTTTACCACTTTGTGCTCTTCCATGTGCACCCGTTCTGGGTCCACCTCTTGTACTTCCTTTCCATCTCCGTCTTGGGT 120
Allel-3         ATGCCCCCTTTGCACGTCCCTAGAAGTAGTAGTCCTCGCAACTTTTACCACCTTCTGCTCTTCCATGTGCACCCGTTCTGGGTCCGACTCTTGTACTTCCTTTCCATCCCCGTCTTGGGT 120
Allel-4         ATGCCCCCTTTGCACGTCCCTAGAAGTAGTAGTCCTCGCAAGTTTTACCACCTTCTGCTCTTCCATGTGCACCCGTTCTGGGTCCAACTCTTGTACTTCCTTTCCATCTCCGTCTTGGGT 120
                *********************** ********* ******* ********* ** ******************************  ********************* ***********

Allel-1         TTCTTGATGCTGAAAGCTCTGCCCATGAAGTCCGGCTCCGTCCCTAGTCCCTCGGCTCTCGACCTGGTCTTCACGTCCGTGTCAGCGACGACAGTCTCCAGCATGGTCGCCGTCGAGATG 240
Allel-2         TTCTTGATGCTGAAAGCTCTGCCCATGAAGTCCGGCTCCGTCCCTAGGCCCTCGGCTCTCGACCTGGTCTTCACGTCCGTGTCAGCGACGACAGTCTCCAGCATGGTCGCCGTCGAGATG 240
Allel-3         TTCTTGATGCTTAAAGCTCTGCCCATGAAGTCCGGCTCCCTCCCTAGGCCCTCGGCTCTCGACCTGGTCTTCACGTCCGTGTCGGCGACGACAGTCTCCAGCATGGTCGACGTCGAGATG 240
Allel-4         TTCTTGATGCTGAAAGCTCTGCCCATGAAGTCCGGCTCCCTCCCTAGGCCCTCGGCTCTCGACCTGGTCTTCAAGTCCGTGTCGGCGACGACAGTCTCCAGCATGGTCGCCGTTGAGATG 240
                *********** *************************** ******* ************************* ********* ************************* *** ******

Allel-1         GAATCCTTCTCCAACTCCCAGCTCCTCCTCATGACCCTCCTGATGCTCCTCGGAGGCGAGGTGTTCACAAGCATGCTTGGTCTCCACTTCACCTGCACCAAGCTCATCAAGAAGAGAGAG 360
Allel-2         GAATCCTTCTCCAACTCCCAGCTCCTCCTCATGACCCTCCTGATGCTCCTCGGAGGCGAGGTGTTCACAAGCATGCTTGGTCTCCACTTCACCTGCACCAAGCTCATCAAGAAGAGAGAG 360
Allel-3         GAATCCTTCTCCAACTCCCAGCTCCTCCTCATGACCCTCCTGATGCTCCTCGGGGGCGAGGTGCTCACAAGCATGCTTGGTCTCCACTTCACCTGCACCAAGCTCATCAAGAAGAGAGAA 360
Allel-4         GAATCCTTCTCCAACTCCCAGCTCCTCCTCATGACCCTCCTGATGCTCCTCGGGGGCGAGGTGTTCACAAGCATGCTTGGTCTCCACTTCACCTGCACCAAGCTCATCAAGAAGAGAGAA 360
                ***************************************************** ********* *******************************************************

Allel-1         ACACCTCATAGTAATAATAGTCTCCATGGTAATATGAATGAACCGCCACCGGCATCTAGTAGCTTAGAGCACAGTCGCCGTCGCCGTCCCATGGAGATGGAAGCCCAGGCCGCCGCCGTC 480
Allel-2         ACACCTCATAGTAATAATAGTCTCCATGGTAATATGAATGAACCGCCACCGGCATCTAGTAGCTTAGAGCACAGTCGCCGTCGCCGTCCCATGGAGATGGAAGCCCAGGCCGCCGCCGTC 480
Allel-3         ACACCCCATAGTAACAATAGGCTCCATGGTAATATTAATGGACCG---CCGGCATCTAGTAGCTTAGAGCACAGTCGCCCTCGCCGTCCCATGGAAATGGAAGCCGGCCAGGCCGCCGTC 477
Allel-4         ACACCTCATAGTAACAATAGGGTCCATGGTAATATTAATGGACCGCCGCCGGCATCTAGTAGCTTAGAGCACAGTCGCCCTCGCCGTCCCATGGAAATGGAAGCCGGCCAGGCCGCCGTC 480
                ***** ******** *****  ************* **** ****   ******************************* *************** *********      *********

Allel-1         CAAATGGAGCTAGCAGGGTTCAACA------------AGGATTTCGCATCCATGGCTAGGCTGCTAATGTGCATCGTGCTGGGCTACCTTGTGGTGGTGCACCTCGCCGGCTACACACTC 588
Allel-2         CAAATGGAGCTAGCAGGGTTCAACA------------AGGATTTCGCATCCATGGCTAGGCTGCTAATGTGCATCGTGCTGGGCTACCTTGTGGTGGTGCACCTCGCCGGCTACACGCTC 588
Allel-3         CAAATGGAGCTAGCAGGGTTCAACGAGAACGACCAACGTGATTTCGCATCCATGGCTAGGCTGCTAATGTTCATCGTGCTGGGCTACCTCTTGGTGGTGCACCTCGCCGGCTACACGCTC 597
Allel-4         CAAATGGAGCTAGCAGGGCTCAACGAGAACGACCAACGTGATTTCGCATCCATGGCTAGGCTGCTAATGTTCATCGTGCTGGGCTACCTCTTGGTGGTGCACCTCGCCGGCTACACGCTC 600
                ****************** *****               ******************************* ******************  ************************* ***

Allel-1         ATACTAATCTACCTCAGCGTGGTCGGCAGCGCGAGAGCAGTGCTGGTTGGCAAGAGGATCAGCCTGAGAACCTTCTCCGCCTTCACGGTCGTCTCGACGTTCGCAAACTGTGGGTTCGTG 708
Allel-2         ATACTAATCTACCTCAGCGTGGTCGGCAGCGCGAGAGCAGTGCTGGTTGGCAAGAGGATCAGCCTGAGAACCTTCTCCGCCTTCACGGTCGTCTCGACGTTCGCAAACTGTGGGTTCGTG 708
Allel-3         ATTCTAATCTACCTCGGCGTGGTCGGCGGCGCGAGAGCAGTGCTGGTTGGCAAGAGGATCAGCCTGAGCACCTTCTCCGTCTTCACGGTCGTCTCGACGTTCGCGAACTGTGGGTTCGTG 717
Allel-4         ATTCTAATCTACCTCGGCGTGGTCAGCGGCGCGAGAGCAGTGCTGGTTGGCAAGAGGATCAGCCTGAGCACCTTCTCCGTCTTCACGGTCGTCTCGACGTTCGCGAACTGTGGGTTCGTG 720
                ** ************ ******** ** **************************************** ********** *********************** ****************

Allel-1         CCAACGAACGAGGGGATGGTGTCCTTCAAGTCCTTCCCGGGCATGCTCCTCCTCGTCATGCCGCACATCCTCCTCGGGAACACGCTCTTCCCCGTCTTCCTCAGGCTCTCCATTACAGCG 828
Allel-2         CCAACGAACGAGGGGATGGTGTCCTTCAAGTCCTTCCCGGGCATGCTCCTCCTCGTCATGCCGCACATCCTCCTCGGGAACACGCTCTTCCCCGTCTTCCTCAGGCTCTCCATTACAGCG 828
Allel-3         CCAACGAACGAGGGGATGGTGTCGTTCAAGTCCTTCCCGGGCATGCTCCTCCTCGTCACCCCGCACATCCTCCTCGGGAACACGCTCTTCCCCGTCTTCCTCAGGCTCTCCATTACAGCG 837
Allel-4         CCAACGAACGAGGGGATGGTGTCCTTCAAGTCCTTCCCGGGCATGCTCCTCCTCGTCATGCCGCACATCCTCCTCGGGAACACGCTCTTCCCCGTCTTCCTCAGGCTCTCCATTACAGCG 840
                *********************** **********************************  ************************************************************

Allel-1         CTCGAGAGAGTCACCAGGAGGCGAGACCTCCGCGAGCTGCTGAAGGACGGAGGACCCGGCGGCGGCGGACCAGCTGCCGCCGCTATAGGCTACGACCACCTGCTGCCTGGAGCCCGCACA 948
Allel-2         CTCGAGAGGGTCATCAGGAGGCGAGACCTCCGCGAGCTGCTGAAGGACGGAGGACCCGGCGGCGGCGGACCAGCTGCCGCCACTATAGGCTACGACCACCTGCTGCCTGGAGCCCGCACA 948
Allel-3         CTCGAGAGGGTCACCAGGAGGCGAGACCTCCGCGAGCTGCTGAAGGACGGAGGACCCGGCGGCGGCGGACCAGCTGCCGCCGCTATAGGCTACGACCACCTGCTGCCCGGAGCCCGCACA 957
Allel-4         CTCGAGAGGGTCACCAGGAGGCGAGACCCCCGCGAGCTGCTGAAGGACGGAGGACCCGGCGGCGGCGGACCAGCTGCCGCCGCTATAGGCTACGACCACCTGCTGCCCCGAGCCCGCACA 960
                ******** **** ************** **************************************************** *************************  ***********

Allel-1         TGGTTCCTGGCTCTCACCGTGGCGGTGTTCCTGGCGGTGCAGCTGCTGCTCTACTGCGCCATGGAGTGGGGCTCCGATGGGCTTCGTGGGCTCACCGCGTTCCAGAAGCTCGTTGCGGCG 1068
Allel-2         TGGTTCCTGGCTCTCACCGTGGCGGTGTTCCTGGCGGTGCAGCTGCTGCTCTACTGCGCCATGGAGTGGGGCTCCGATGGGCTTCGTGGGCTCACCGCGTTCCAGAAGCTCGTTGCGGCG 1068
Allel-3         TGGTTCCTGGCTCTCACCGTGGCGGTGTTCCTGGCGGTGCAGCTGCTGCTCTACTGCGCCATGGAGTGGGGCTCCGATGGGCTTCGTGGGCTCACCGCGTTCCAGAAGCTCGTTGCGGCG 1077
Allel-4         TGGTTCCTGGCTCTCACCGTGGCGGTGTTCCTGGCGGTGCAGCTGCTGCTCTACTGCGCCATGGAGTGGGGCTCCGATGGGCTTCGTGGGCTCACCGCGTTCCAGAAGCTCGTTGCGGCA 1080
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Allel-1         CTCTTCATGTCCGTCAACTCCAGGCACTCCGGCGAGATGGTCGTCGACCTCGCCACCGTGTCGTCCGCCATCGTCGTGCTCTATGCGCTCATGATGTAAGTAAAGTACGTACATAAATAC 1188
Allel-2         CTCTTCATGTCCGTCAACTCCAGGCACTCCGGCGAGATGGTCGTCGACCTCGCCACCGTTTCGTCCGCCATCGTCGTGCTCTATGTGCTCATGATGTAAGTAAAGTACGTACATAAATAC 1188
Allel-3         CTCTTCATGTCCGTCAACTCCAGGCACTCCGGCGAGATGGTCGTCGACCTCGCCACCGTGTCGTCCACCATCGTCGTGCTCTATGTGCTCATGATGTAAGTAAAGTACGTACATGCATAC 1197
Allel-4         CTCTTCATGTCCGTCAACTCCAGGCACTCCGGCGAGATGGTCGTCGACCTCGCCACCGTGTCGTCCACCATCGTCGTGCTCTATGTGCTCATGATGTAAGTAAAGTACGTACATAA---- 1196
                *********************************************************** ****** ****************** ****************************

Allel-1         ATCGATCGAT----ATTCGATATATAGAAGATCCAAGATC----GTCGGTAGTGTTAATTAGGAAGTTTTGTGCACTACATTTATTATATTATATTTAGACATATGACATCAGGCACTGC 1300
Allel-2         ATCGATCGAC-GATATTCGATATATAGAAGATCCAAGATCGTCGGTACGTAGTGTTAATTAGGAAGTTTTGTGCACTACATTTATTATATTATATTTATACATATGACATCAGGCACTGC 1307
Allel-3         ATACATCGTTCGATATTCGATATATAGAAGATCCAAGATC----GTCGGTAGTGTTAATTAGGAAGTTTTGTGCACTACATTTATTATATTATATTTATACATATGATACCAGGCACTGC 1313
Allel-4         ATACATCGATCGATATTCGATATATAGAAGATCCAAGATC----GTCGGTAGTGTTAATTAGGATGTTTTGTGCACTACATTTATTATAC-----------ATATGACATCAGGCACTGC 1301
                **  ****      **************************    **  **************** ************************            ****** * **********

Allel-1         TATCTAGTTCATGCAT----AGTTAGTTGCACATGTGCTCATGGTCCACCAACACGTCCAAATATCGATTTGTTATAATATATAGTTTGATCACGTTCCAACAATATGTATTGATATTAC 1416
Allel-2         TATCTAGTTCATGCAT----A----GTTGCACATGTGCTCATGGTCCACCAACACGTCCAAATATCGATTTGTTATA--ATATAGGTTGATCACGTTCCAACAATATGTATTGATATTAC 1417
Allel-3         TATCTAGTTCATGCATGGTTAATTAGTTGCACATGTGCTCATGGTCCACCAACACACGTTCAAATATCGATTTGTTATAATATAGTTTGATCACGTTCCAACAATATGTATTGATATTAC 1433
Allel-4         TATCTAGTTCATGCAT----AGTTAGTTGCACATGTGCTCATGGTCCACCA--ACACGTCCAAATATCGATTTGTTATAATATAGTTTGATCACGTTCCAACAATATGTATTGATATTAC 1415
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Allel-1         TACTGATACTAAATGATATAGCCTTACTTGAAACTTATCATCGTGTCATGCGTATGACTATATGATCATGACACATGATGTTTAAGCAGTGTTTTAAATAGCCAGCTATGGCATTTAGCT 1536
Allel-2         TACTAATACTAAATGATATAGCCTTACTTGAAACTTATCATCGTGTCATGCGTATGGCTATATGATCATGACACATGATGTTTAAGCAGTGTTTTAAATAGCCAGCTAAGGCATTTAGCT 1537
Allel-3         TACTAATACTAAATGATATAGCCTTACTTGAAACTTATCATCGTGTGATGCGTATGACTATATGATCATGACACATGATGTTTAAGTAGTGTTTTAAATAGCCAGTTAAGGCATTTAGCT 1553
Allel-4         TCCTAATACTAAATGATATAGCCTTACTTGAAACTTATCATCGTGTGATGCGTATGACTATATGATCATGACACATGATGTTTAAGCAGTGTTTTAAATAGCCAGCTAAGGCATTTAGCT 1535
                * ** ****************************************** ******** ***************************** ****************** ** ***********

Allel-1         ATTTATGTCCCAAAACAGGCTATAGCAGGGTTATAGCGGCAGTTAAGAGCTAAATTGTACGTGAGAAAACTTAGCTAAATCATTCTCAAACAGCTATAGCCGGAGATTTAAAACCTTGTG 1656
Allel-2         ATCTATGTCCCAAAATAGGCTATAG-----------CGGCGACTAAGAGCTAAATTGTACATGAGAAAACTTAGCTAAATCCTTCTCAAACAGCTATAGCCGGAGATTTAAAACCTTGGG 1646
Allel-3         ATCTATGTCCCAAAACAAGCTATAGCAGAGCTATAGCGGCAGCTAAAAGCTAAATTGTACATGAGAAAACTTAGCTAAATCCTTCTCAAACAGCTATAGCCGGAGATTTAAAACCTTATG 1673
Allel-4         ATCTATGTCCCAAAACAAGCTACAACAGGGCTATAGCGGTAGTTAAGAGCTAAATTGTACATGAGAAAACTTAGCTAAATCCTTCTCAAACAGTTATAGCTGGAGATTTAAAACCTTGTG 1655
                ** ************ * **** *            ***    *** ************* ******************** *********** ****** ****************  *

Allel-1         TTTAAGTTAGCAAGCATACAAACTATTACTAGTTTTTTTTTCTTTAAATAATTTTACTCGGTTATGCTCATGCTTTACTTCCTCTGGCAGGTATCTACCGCCTTACACCACATTTTTGCC 1776
Allel-2         TTTAAGCTAGCAAGCATACAAACTATTACTATTTTT--TTTCTTTAAATAATTTTACTCGGTTATGCTCATGCTTTACTTCCTCT-GCAGGTATCTACCGCCTTACACCACATTTTTACT 1763
Allel-3         TTTAAGCTAACAAGCATACAAACTATTACTAGTTTTTTTTTCTTTAAATAATTTTACTCGGTTATGCTCATGCTTTACTTCCTCT-GCAGGTATCTACCGCCTCACACCACATTTTTACC 1792
Allel-4         TTTAAGCTATCAAGCATACAAACTATTACTAGTTTTT-TTTCTTTAAATAATTTTACTCGGTTATGCTCATGCTTTACTTCCTCT-GCAGGTATCTACCGCCTTACACCACATTTTTACC 1773
                ****** ** ********************* ****  ************************************************ **************** ************* *

Allel-1         CGTAGCGATGGAAGACCAGCAGCAGCAGCAAAATGAGGCACAACCCGACGACAAGAGCAAGAGCAGCAGCAGTATTAGCATCTGGCAGAAGCTGCTCATGTCGCCGCTCTCGTGCCTTAC 1896
Allel-2         CGTAGCGATGGAAGACCAGCAGCAGCAGCAAAATGAGGCACAACCCGACGACAAGAGCAAGAGCAGCAGCAGTATTAGCATCTGGCAGAAGCTGCTCATGTCGCCGCTCTCGTGCCTAAC 1883
Allel-3         CGTAGCGATGGAAGACCAGCAGCAGCAGCAAAATGAGGCACAACCCGACGACAAGAGCAAGAGCAGCAGCAGTATTAGCATCTGGCAGAAGCTGCTCATGTCGCCGCTCTCGTGCCTAAC 1912
Allel-4         CGTAGCGATGGAAGACCAGCAGCAGCAGCAAGATGAGGCACAACCCGACGACAAGAGCAAGAGCAGCAGCAGTATTAGCATCTGGCAGAAGCTGCTCATGTCGCCGCTCTCGTGCCTAAC 1893
                ******************************* ************************************************************************************* **

Allel-1         CATCTTCATCGTCGTCATCTGCATCACCGAGCGGCGGCAGATTGCTCACGACCCAATCAACTTCAGCGTCCTCAACATCGTCATCGAAGTCATCAGGTGTTTATTAATTAATCAATTAAC 2016
Allel-2         CATCTTCATCGTCGTCATCTGCATCACCGAGCGGCGGCAGATTGCTCACGACCCAATCAACTTCAGCGTCCTCAACATCGTCGTCGAAGTCATCAGGTGTTTATTAATTAATCAATTAAC 2003
Allel-3         CATCTTCATCGTCGTCATCTGCATCACCGAGCGGCGGCAGATTGCTCACGACCCAATCAACTTCAGCGTCCTCAACATCGTCGTCGAAGTCATCAGGTGTTTATTAATTAATCAATTGAC 2032
Allel-4         CATCTTCATCGTCGTCATCTGCATCACCGAGCGGCGGCAGATTGCTCACGACCCAATCAACTTCAGCGTCCTCAACATCGTCGTCGAAGTCATCAGGTGTTTATTAATTAATCAATTAAC 2013
                *********************************************************************************** ********************************* **

Allel-1         TCCAAGCGCAATAGATGATCTCTTGCACTGCACTGGGCTCACAATGAAGCAATGCAACATGGTGATGCGTGCAGTGCGTATGGCAACGTGGGATTCAGCACCGGGTACAGCTGCAGCAGG 2136
Allel-2         TCCAAGCGCAATAGATGATCTCTTGCACTGCACTGGGCTCACAATGGAGCAATGCAACATGGTGATGCGTGCAGTGCGTATGGCAACGTGGGATTCAGCACCGGGTACAGCTGCAGCAGG 2123
Allel-3         TCCAAGCGCAATAGATGATCTCTTGCACTGCACTGGGCTCACAATGAAGCAATGCAACATGGTGATGCGTGCAGTGCGTATGGCAACGTGGGATTCAGCACCGGGTACAGCTGCAGCAGG 2152
Allel-4         TCCAAGCGCAATAGATGATCTCTTGCACTGCACTGGGCTCACAATGGAGCAATGCAACATGGTGATGCGTGCAGTGCGTATGGCAACGTGGGATTCGGCACCGGGTACAGCTGCAGCAGG 2133
                ********************************************** ************************************************* ***********************

Allel-1         CAGGTGACGCCCGACGGCAGCTGCAGGGACGCGTGGGTTGGCTTCTCCGGCAAGTGGAGCAGGGAAGGGAAGCTCACGCTCATGGCCATCATGTTCTACGGCCGGCTCAAGAAGTCCAGC 2256
Allel-2         CAGGTGACGCCCGACGGCAGCTGCAGGGACGCGTGGGTTAGCTTCTCCGGCAAGTGGAGCAGGGAAGGGAAGCTCACGCTCATGGCCGTCATGTTCTACGGCAGGCTCAAGAAGTTCAGC 2243
Allel-3         CAGGTGACGCCCGACGGCAGCTGCAGGGACGCGTGGGTTGGCTTCTCCGGCAAGTGGAGCAGGGAAGGGAAGCTCACGCTCATGGCCGTCATGTTCTACGGCAGGCTCAAGAAGTTCAGC 2212
Allel-4         CAGGTGACGCCCGACGGCAGGTGCAGGGACGCGTGGGTTGGCTTCTCCGGCAAGTGGAGCAGGGAAGGGAAGCTCACGCTCATGGCCGTCATGTTCTACGGCAGGCTCAAGAAGTTCAGC 2253
                ******************** ****************** *********************************************** ************* ************* ****

Allel-1         ATGCATGGGGGTCAGGCATGGAAGCTAGGCTAA 2289
Allel-2         ATGCATGGGGGTCAGGCATGGAAGCTAGGCTAA 2276
Allel-3         ATGCATGGGGGTCAGGCATGGAAGCTAGGCTAA 2305
Allel-4         ATGCATGGGGGTCAGGCATGGAAGCTAGGCTAA 2286
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Figure 1. DNA sequences of 4 alleles of HKT1;5 present in *Miscanthus* genotypes OPM-37 and OPM-48.

2.2.3 Cl^- homeostasis

Both Cl^- and Na^+ accumulate in the plants under salt stress but the mechanisms of Cl^- transport are less well understood, compared with K^+ and Na^+. In our experiments, there was a strong relationship between Cl^- and Na^+ at different salt stress levels (150 and 250 mM NaCl) in pots and in hydroponics system (150 mM NaCl). This agrees with the fact that plants under salt stress need to keep electrochemical balance, and Cl^- and Na^+ uptake are often linked (Teakle and Tyerman, 2010). Under salt stress, the leaf Na^+ contents were generally lower than the leaf Cl^- contents in *Miscanthus*, and the root Na^+ contents were higher than the leaf Cl^- contents. This indicates that most *Miscanthus* genotypes in our test set have an active Na^+ exclusion mechanism, but do not utilize a Cl^- exclusion mechanism under these conditions, and that under these stress conditions, Na^+ and Cl^- are also not strictly electrochemically linked. Generally, the Cl^- toxicity levels are considered to be 4-7 mg/g DW for Cl^- sensitive species and 15-50 mg/g DW for Cl^- tolerant species (Xu et al., 1999). The average leaf Cl^- content was 18 (mg/g DW) at 150 mM NaCl in hydroponics and 20.7 (mg/g DW) in *in-vitro* started plants and 23.7 (mg/g DW) in rhizome-based plants at 250 mM NaCl in pots, indicating that the Cl^- contents may reach toxic levels, and affect the performance of the plants, and indeed genotype OPM-9 and 71 showed serious senescence, and contained over 30 mg/g DW Cl^- in the leaves. Several chloride channel (CLC) genes were cloned from *Arabidopsis* and a functional association of these genes with Cl^- homeostasis was identified (Lv et al., 2009). Overexpressing *ZmCLC-d* in *Arabidopsis* improved salt tolerance and reduced accumulation of Cl^- in transgenic plants as compared with wild type plants (Wang et al., 2014). Further identification and characterization of the genes involved in Cl^- uptake, transport and storage will further contribute to breeding programs aimed at improving salt tolerance and combustion quality in *Miscanthus* due to corrosive issues of high Cl^- contents (Lewandowski et al., 2003).

2.2.4 Ca^{2+} homeostasis

Na^+ strongly competes with Ca^{2+} uptake to the shoots under saline stress (Liu and Zhu, 1998). High soil salinity thus can interfere with Ca^{2+} uptake and nutrition, which may cause Ca^{2+}-deficiency in salt-stressed plants. This is the reason why it is suggested that adding supplemental Ca^{2+} to the hydroponics media is necessary to discriminate between the effect of Na^+ and Ca^{2+} deficiency induced by high concentrations of Na^+ (Munns and Tester, 2008; Genc et al., 2010). Ca^{2+} uptake was also decreased by salt stress in wheat, increasing Na^+/Ca^{2+} ratios, which caused a reduction in growth and yield (Perveen et al., 2012). In our hydroponics system, we did not add extra Ca^{2+} to compensate for the effects of high Na^+ on Ca^{2+} uptake and utilization. The concentrations of Ca^{2+} measured in the leaf tissues are above 4.5 mg/g DW at 150 mM NaCl in hydroponics and above 5.12 mg/g DW at 250 mM NaCl in pots, which is higher than the proposed minimum threshold (around 1 mg/g DW) for Ca^{2+}-deficiency in leaf tissues (Genc et al., 2010). Moreover, the field trials (Chapter 5) revealed no significant difference in Ca^{2+} contents between EC value of 0-6 dS/m and above 6 dS/m. For other crops such as wheat and rice, salt stressed plants had a considerable reduction in Ca^{2+} content in comparison to control conditions (Hakim et al., 2014), and some salt tolerant wheat varieties have a better ability to maintain stable Ca^{2+} levels in both shoots and roots (Perveen et al., 2012; Hakim et al., 2014). Ca^{2+} is acting as a second messenger in stress signaling and salt stress induces changes in Ca^{2+} for rapid long-distance root-to-shoot signaling in plants, regulating ion homeostasis (Choi et al., 2014). Salt stress leads to a cytosolic Ca^{2+}-signal that activates the calcium sensor protein SOS3. SOS3 activates and binds to the protein kinase SOS2. This SOS3/SOS2 complex regulates the activities of a plasma membrane Na^+/H^+ antiporter encoded by *SOS1* gene. This results in either Na^+ efflux out of the cytosol or its compartmentation in the vacuole, which helps plants to cope with Na^+ accumulation under stress conditions (Hadi and Karimi, 2012). It is interesting to note that *Miscanthus* maintains similar Ca^{2+} contents in leaf tissues under both salt stress and control conditions in pots, and in the field (Chapter 3, 4, and 5). Since Ca^{2+} regulates genes involved in the salt stress response, maintaining relatively high Ca^{2+} concentrations may be a mechanism contributing to *Miscanthus* salt tolerance and may be one of the reasons why *Miscanthus* is classified to moderate salt tolerance.

3 Breeding multiple-tolerance *Miscanthus* genotypes for marginal lands in the future

Since *Miscanthus* is expected to be grown on marginal lands to avoid competition with food crops and will therefore likely be exposed to more than one stress, it is important to breed multiple stress-tolerant *Miscanthus* genotypes (Lewandowski et al., 2016). Marginal conditions may combine extremes of temperature with drought stress and possibly salt stress (Mittler, 2006). Because exposure to abiotic stresses reduces the yield below their potential, it is a main barrier to expand bioenergy crops to marginal lands if the yields are too low to achieve an economic viable level. This is exemplified by the low yields of the genotypes grown in the Dongying field trial described in Chapter 5, where in addition to salt stress, the plants were likely exposed to nutrient limitation and occasional drought. Thus, in addition to salt stress tolerance, we need to identify and evaluate the different physiological traits under various other stresses as well as combined multiple stresses to achieve combined stress tolerance for growth in marginal areas, for instance by crossing the highly tolerant genotypes to single stress resistant lines to produce multiple stress-tolerant hybrids.

Plants may need to use different mechanisms to promote tolerance and survival under different stresses. Although many mechanisms are likely to be stress specific, metabolic shifts that reallocate resources and transmit signals to active stress-specific mechanisms represent a pathway that may occur under many types of adverse conditions (Mittler, 2006; Swindell, 2006). QTL studies indeed have identified genetic correlations among stress tolerance traits, and certain heat-shock proteins are commonly elicited in response to various stress conditions (Givskov et al., 2003). Selection for tolerance to one type of stress has been associated with tolerance to another type of stress (Agrawal et al., 2004). Transcription factors (DREB), calcium-dependent signaling and the phytohormone abscisic acid (ABA) can be induced by drought, salinity, and extreme temperature in the *Arabidopsis* (Liu et al., 1998; Jin-Baek et al., 2004; Huang et al., 2011). The associations of the responses between different abiotic stresses were analyzed in *Arabidopsis* and the highest average correlation (0.565) in shoots exists between salt and osmotic stress, medium correlation (0.275) between salt and cold stress, while the lowest average correlation (0.196) in shoots was found between drought and cold stress. It is therefore possible that the highly salt tolerant *Miscanthus* genotypes also have tolerance to other stresses. However, one stress factor may also compromise tolerance to another, and stress tolerances to different stresses are certainly not always additive (Kissoudis et al., 2014).

3.1 Drought tolerance and salt tolerance

Water may be limitedly available in marginal areas, and drought will negatively affect crop yield and quality. Moreover, drought stress also increases soil salinity levels because less water is available to leach salts to the environment, and under high evaporation conditions, drought may occur while minerals are increasingly deposited in the upper soil layers (Rengasamy, 2006). Salt stress is an increasing problem especially for irrigation agriculture and soil salinity is further increasing because of poor drainage and climate change (Rengasamy, 2010). Since bioenergy crops are required to produce sustainable and economically viable biomass under low input conditions including limited irrigation, breeding genotypes that are both drought-tolerant and salt-tolerant is important. Osmotic tolerance may be helpful under drought stress as well as salt stress. Although *Miscanthus* × *giganteus* possesses a range of desirable traits and is higher yielding than its parents, it was found to be less drought tolerant then its parent species, in particular *M. sinensis* (Clifton-Brown and Lewandowski, 2000b). The genotypic variation of *Miscanthus* for drought tolerance was assessed in a large-scale screen with 49 genotypes in a pot experiment and several potential *Miscanthus* ssp exceeded *Miscanthus* × *giganteus* yield both under control and drought conditions (van der Weijde et al., 2016b). Genotype OPM-31 demonstrated the highest drought tolerance, followed by OPM-44, 98, 91, 46, 67, and 6 (van der Weijde et al., 2016b). In addition, genotype OPM-6 and OPM-98 showed higher yield than *Miscanthus* × *giganteus* under both control and drought (van der Weijde et al., 2016b). Therefore, these two genotypes were considered as potential materials for breeding and starting materials for field cultivation with limited irrigation. Interestingly, genotype OPM-31 showed not only the highest drought tolerance, but also the highest salt tolerance in hydroponics screen (Chapter 1 Figure 4). In hydroponics, genotype OPM-31 did not show a significant difference in leaf expansion rate between salt stress and control conditions, indicating that this genotype may be tolerant to osmotic stress under both drought and salt stress conditions. Similarly, genotype OPM-67 may also be tolerant to osmotic stress as it was ranked 6th in drought tolerance in 49 tested genotypes and 8th in salt tolerance of 70 tested genotypes. Genotypes OPM-31 and OPM-67 therefore may have potential to improve osmotic tolerance in salt and drought lands. To some extent, genotype OPM-67 with the highest salt tolerance of the 12 tested genotypes in this pot experiment also utilized Na^+ exclusion mechanisms to actively keep the ions from accumulating in the leaves, thus minimizing damage to essential physiological processes such as photosynthesis and maintaining higher yield than genotype OPM-31 under salt stress.

3.2 Cold tolerance and salt tolerance

Low temperature can be highly stressful, or even lethal for plants. For example, frost can kill the rhizomes during winter, or damage newly emerged shoots in spring. Cultivation of bioenergy crops in high latitude regions requires cold-tolerant genotypes (Zub et al., 2012). Especially a perennial such as *Miscanthus* needs to be able to survive after winter when grown in regions with cold winters, or reduce the damage of new shoots by frost in early spring (Clifton-Brown and Lewandowski, 2000a). If the winter mortality rate of *Miscanthus* plants can be decreased by improving rhizome cold tolerance, expansion of the potential growing areas to higher latitudes may be enabled. Genotypic variation for frost tolerance has been reported in a large *Miscanthus* germplasm set that included 117 genotypes representing current European breeding programs (76 *M. sinensis,* 17 *M. sacchariflorus,* 11 *Miscanthus* × *giganteus* and 13 interspecific hybrids), overlapping with the genotypes used in this thesis (Fonteyne et al., 2016). On average, the LT_{50} (the temperature at which 50% of the rhizomes are killed) for *Miscanthus* × *giganteus* was -2.6 ± 0.3°C, -3.5 ± 0.1°C in *M. sinensis*, -2.6 ± 0.3°C in *M. sacchariflorus* and -3.9 ± 0.2°C in the *M. sinensis* × *M. sacchariflor*us hybrids (Fonteyne et al., 2016). A number of the genotypes was more tolerant to frost temperatures than *Miscanthus* × *giganteus* and may be considered as breeding materials to produce new frost tolerant varieties (Fonteyne et al., 2016). The top seven most frost-tolerant *M. sinensis* genotypes were OPM-64, 100, 95, 102, 104, 72, and 84. Interestingly, genotype OPM-72 and OPM-84 were ranked 12th and 15th, respectively, in the salt tolerance evaluation of Chapter 2 (Chen et al., 2017). Both genotype OPM-72 and OPM-84 showed higher Ca^{2+} contents in shoot under control (9.16 and 9.22 mg/g DW, respectively, average =6.9 mg/g DW) and salt stress (6.75 and 7.09 mg/g DW, respectively, average =4.5 mg/g DW). An increase in intracellular Ca^{2+} can activate the expression of transcription factors that enhance cold tolerance in the plants (Rihan et al., 2017). If Ca^{2+} plays an important role in response for both salt and cold stress, these genotypes with high Ca^{2+} may be considered as potential genotypes for multi-stress breeding.

Multi-stress tolerant *Miscanthus* materials for marginal soils are required, and these genotypes should be tested by different screening procedures before planting on marginal lands (Lewandowski et al., 2016). Although it is not straightforward to breed for multi-stress tolerance, the evaluations for different individual stress tolerances indicate that some genotypes showed high potential to produce biomass under different stress conditions. Thus, the next step would be to test the top performers from each stress including cold, drought and salt evaluations in different climatic regions to establish whether these selections are also relatively tolerant under varying field conditions. These stress-tolerant or resilient genotypes may also be crossed with each other, or with highly productive parental lines, and the progeny evaluated by laboratory screens and field trials under different stresses.

4 Breeding aims in future work

4.1 Sterile genotypes reduce the risk of invasive behavior

Invasiveness is a concern for development of *Miscanthus* that can aggressively spread by rhizomes, seeds or nodal growth. *M. sinensis* was observed to spread by viable seeds (Meyer and Tchida, 1999) and *M. sacchariflorus* seems to spread by creeping shoots (Quinn et al., 2010). The triploid sterile *Miscanthus* × *giganteus* is considered to have a lower risk of escape because of sterile seeds and no running shoots. In our study, *Miscanthus* × *giganteus* showed medium salt tolerance in the hydroponic system and low salt tolerance in field trials. Developing new triploid sterile genotypes may reduce the risk of invasiveness into natural habitats. The triploid sterile *Miscanthus* × *giganteus* genotype can be produced by crossing diploid *M. sinensis* and tetraploid *M. sacchariflorus* genotypes. We have identified several highly salt tolerant *M. sinensis* genotypes and *M. sacchariflorus* accessions. Crossing these genotypes and accessions may create superior genotypes for producing biomass on marginal lands. Although it requires interspecies crossing, producing triploid sterile genotypes of *M. sinensis* by intraspecies crossing is easy. Triploid sterile genotypes *M. sinensis* can be produced by crossing diploid genotypes with tetraploid genotypes that have undergone chromosome doubling (Koefoed Petersen et al., 2003). *M. sinensis* is typically diploid, but natural and artificial polyploids are available. For example, the triploid sterile genotype *M. sinensis* 'Goliath' showed similar yield to *Miscanthus* × *giganteus* (Jorgensen and Muhs, 2001). The other advantage is that the pollen fertility rate of diploid *M. sinensis* is high (>86%) with a low rate of self-pollination, so open pollination can reduce the cost of seed production (Yamada, 2015). The identified salt tolerant *M. sinensis* genotypes can be used for chromosome doubling and creation of new triploid hybrids by crossing diploid *M. sinensis* genotypes with complementary or matching traits, such as drought or cold tolerance.

4.2 Seed production vs *in vitro* and rhizome propagation

One of the limitations for expansion of the *Miscanthus* cultivation area is the low propagation rate from rhizomes and high cost for plantation. This limitation would be overcome by develop hybrid seeds if invasiveness risk is low or seed is produced in regions for which *Miscanthus* is a native species. Current seed-based propagation methods can increase the propagation rate 100-fold compared with rhizome propagation (Hastings et al., 2017). In addition, it is estimated that the cheapest way to plant *Miscanthus* could be direct seed sowing compared with seedlings from rhizomes or tissue culture (Xue et al., 2015). Efforts are now made to develop hybrid seeds to upscale the *Miscanthus* crop cultivation area to millions of hectares and reduce planting costs. Recently, the use of hybrid seeds in modular plugs before planting in the field for pre-commercial scale trials has been tested and the field data for yield estimation in recent years will be added (Clifton-Brown et al., 2017). A high seed germination rate is important for plantation, but this is variable because of several factors including matureness at harvest and dormancy (Hastings et al., 2017). Hastings et al. (2017) reported that a hectare of seed production can produce enough seeds to plant ~2,000 ha. However, direct sowing is often unreliable due to the low germination rates, and young seedlings are highly sensitive to soil moisture and needs to compete with weeds (Clifton-Brown et al., 2017). To reduce the risks, seeds can be sown in potting soil in the greenhouse, with day/night temperatures set at 25/18˚C until the seedlings have more than 3 leaves before transplanting. In addition, breeding programs can focus on tolerance to abiotic stresses at an early seedling stage. The results with young vegetative plantlets from our hydroponics salt tolerance evaluation in Chapter 2 may be used to further develop varieties that are tolerant to salt and limited water availability at an early stage of development. Similar experiments in hydroponics targeting nitrogen use efficiency may also help to identify growth vigor at an early stage under limited nutrient supply, comparable to the conditions in marginal soils. The seeds collected from different locations in China were sown under controlled conditions for two months before transplanting (Chapter 5). The transplanted seedlings could survive at an EC value below 17.3 dS/m while the mortality rate was 100% when the EC value was higher than 17.3 dS/m. The field trials in China showed that *Miscanthus* seedlings from seeds from tolerant genotypes on marginal land survive with relatively low mortality (Chapter 5). Once potential genotypes are identified, they may be used as parents that can produce, in different crossing combinations, a collection of hybrid F1 seeds that meet the requirements of different types of marginal lands in the future and accelerate mass selection in plant breeding.

4.3 Molecular breeding

The progress in breeding of perennials, such as *Miscanthus*, is slower than in annual crops because evaluating genotype performance in field trials needs several years. Marker-assisted selection could accelerate breeding efficiency, as marker data can be used to select seedlings at an early stage. The large genome size of *Miscanthus* and its high level of heterozygosity may complicate the use of the genome sequence and molecular markers (Głowacka, 2011). As a result, only a few genetic maps of *Miscanthus* have been published to date (Atienza et al., 2002; Ma et al., 2012; van der Weijde et al., 2017c). Van der Weijde et al. (2017) tried to detect QTLs for biomass composition in field trials through using a genetic map for *M. sinensis* with markers generated by a genotyping-by-sequencing (GBS) approach. A mapping population of 186 F1 progenies from a cross between two *M. sinensis* genotypes contrasting for cell wall composition was grown under field conditions for three years (van der Weijde et al., 2017c). A total of 86 QTLs for a variety of biomass quality characteristics were identified and twenty QTLs were stably detected in both growth seasons (van der Weijde et al., 2017c). We tested a half-sib population of this cross for salt stress tolerance on our hydroponics conditions, using a similar setup as described in Chapter 2, and detected only two significant QTLs involved in the *Miscanthus* salt stress response. One genetic region was linked to the salt-dependent reduction in plant height (control/salt) and the other to the salt-induced reduction in root dry weight (control/salt). The low number of QTLs may be due to the low resolution and relatively low quality of the genetic map, and increasing the number of genetic markers may enhance resolution. Additionally, the two parental lines were selected based on contrasting cell wall properties, and not for salt tolerance. The three half-sib (grand) parental families indeed did not demonstrate a lot of variation in salt tolerance (37% - 41.7%) in a hydroponic system.

4.4 Greenhouse gas emissions of bioenergycrops

Finally, it may be important to (re)consider the environmental costs and benefits of bioenergy, which has been the subject of significant debate. Some studies (particularly on transport fuels) have indicated that bioenergy systems may even increase greenhouse gas (GHG) emissions (Smith Keith and Searchinger Timothy, 2012; Smith et al., 2013), while others showed bioenergy can reduce GHG emissions through life cycle assessment (LCA) studies, a well-established tool used to calculate the environmental impact of a product across a range of impact factors, including climate impacts, as compared to that of the conventional fossil (Thornley et al., 2009; Thornley et al., 2015). In addition to lower life-cycle GHG emissions, perennial crops have other environmental benefits compared with annual crops. For instance, perennial crops such as *Miscanthus* have low nitrogen input requirements, which limits N_2O emission, and they contribute to soil carbon due to reduced tillage and increased belowground biomass allocation (Hudiburg et al., 2015; Carvalho et al., 2017) However, a wide range of factors including climate, soil texture, previous crops, and field management will cause uncertainties in the life-cycle GHG balance of bioenergy crops (Rowe et al., 2011; Sylvestre and Reinhart, 2012; C. et al., 2013; Davis Sarah et al., 2013). Therefore, developing suitable field management strategies for different types of lands along with improvement of varieties to match environmental requirements is necessary to make bioenergy an important tool to mitigate climate change (Jeanette et al., 2018).

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Summary

Miscanthus is a perennial grass with a C4 photosynthetic pathway and characterized by high productivity and resource use efficiency, which makes it favorable for bioenergy production. *Miscanthus* is utilized mostly for direct combustion, or for anaerobic digestion to produce biofuel such as biomethane or bioethanol. In order to avoid competition with food crops, *Miscanthus* will need be grown on underutilized marginal lands like saline soils. Almost 20% of the world's irrigated agriculture is affected by salinity and soil salinity is further increasing due to poor drainage and climate change. Significant crop yield losses and quality reduction are observed due to salinity. Salinity stress includes osmotic stress, ionic stress and nutritional imbalance, and breeding for salt tolerance is not straightforward due to its genetic complexity. Extending *Miscanthus* cultivation further to marginal areas and maintaining economically viable yields and quality depend on suitable genotypes, and evaluation of the performance of *Miscanthus* germplasm under salt stress and identifying salt tolerant genotypes for cultivation on saline soils or as breeding parents to improve salt tolerance is essential for sustainable bioenergy production. This thesis applies several screening systems and experimental approaches to identify genetic variation for salt tolerance, elucidate the salt tolerance mechanisms underlying this variation in *Miscanthus* and to identify the potential genotypes for breeding or direct production on marginal lands.

In Chapter 2, seventy *Miscanthus* genotypes were screened in a hydroponics system in the greenhouse to identify genotypic variation for salt tolerance. Analyses of shoot growth traits and ion concentrations revealed the existence of variation for osmotic tolerance and ionic tolerance in the genotypes. Some genotypes appeared to utilize osmotic tolerance mechanisms to maintain a low reduction in growth and biomass under saline conditions. Several relatively salt tolerant genotypes had clearly lower Na^+ concentrations in the shoots under salt stress, indicating that a Na^+ exclusion mechanism was utilized to prevent Na^+ accumulation and ion toxicity in the leaves. The genotypes demonstrating different salt tolerance mechanisms may serve as starting materials for improving salinity tolerance of *Miscanthus*. Twelve genotypes of *Miscanthus* (11 genotypes that were salt tolerant and/ had high yield on the hydroponics systems in Chapter 2 and genotype OPM-9, *Miscanthus* × *giganteus* as the reference), were evaluated for salt tolerance and ion contents under three salinity levels (0, 150, and 250 mM NaCl) in pots in Chapter 3. Additionally, two types of starting material: *in vitro*-started plants and rhizome-based plants, were used in this study for exploring the influence of the rhizome on salt tolerance. A salinity level of 150 mM NaCl caused a

significant but small reduction of shoot biomass, and hardly any accumulation of ions in leaves, but the effects of salt and genotypic differences were more evident at 250 mM NaCl, with varying levels of Na^+ and Cl^- accumulation in leaves. At both salt levels, plants started from rhizomes had less senescence symptoms than *in vitro*-started plants, without significant differences in ion contents in shoots, indicating that the rhizomes may have the ability to delay senescence, possibly by osmotic adjustment or reducing damage from reactive oxygen species (ROS). The *M. sinensis* genotype OPM-56 had both low Na^+ and Cl^- contents in the shoots and relatively high yield compared to the other genotypes under both saline and normal growth conditions. Therefore, genotype OPM-56, with an effective Na^+ exclusion mechanism to maintain low ion contents in shoots, was considered to have high potential to be grown on saline soils and as a starting material for breeding salt tolerant *Miscanthus* varieties.

In addition to yield, we also evaluated *Miscanthus* biomass quality under salt stress in Chapter 4. The composition of the cell wall is important in relation to *Miscanthus* utilization for combustion or biofuel, and this may be affected by salt stress. In a pot experiment at 150 mM NaCl salt stress, Na^+ ions hardly accumulated in the shoots after 6 weeks of stress, and an increase in senescence indicated that the plants were mostly experiencing osmotic stress. Analyses of cell wall composition in the stems of the plants revealed small changes in cell wall composition, with an increase in hemicellulose content and a reduction in cellulose, which may positively affect cell wall degradability. However, when *Miscanthus* was exposed to more severe stress conditions (200 mM NaCl) and harvested after the growing season (after 19 weeks of salt stress), Na^+ and Cl^- contents in the shoots were significantly increased, and the leaf hemicellulose content decreased while leaf lignin content increased. This change in cell wall composition may have a negative effect on cell wall degradability because lignin inhibits enzymatic deconstruction of the cell wall. Genotypes OPM-48 and 87 had higher hemicellulose and lower lignin content than the other genotypes under salt stress, and are candidates for biogas production on marginal soils.

In addition to experiments under controlled conditions within one growth cycle, a 2-year field trial was conducted on marginal lands in China (Chapter 5). A collection of twenty *M. sacchariflorus* accessions from diverse locations in China and a set of five genotypes fom Aberystwyth University, UK were estimated for their potential yield under marginal field conditions in Dongying, China. On average, the entire *Miscanthus* set demonstrated 43% salt tolerance (biomass salt/control) at EC 6 dS/m in the second growing year. At this salinity level, accessions collected in Dongying, China showed the highest yield under salt stress but also under control conditions, and the accession from Haizhou, China showed the highest salt tolerance. Two accessions collecting from coastal areas demonstrated the best performance under moderately salinity conditions (EC 4-8 dS/m), suggesting that accessions growing in coastal areas, with a higher incidence of salinity, may be valuable resources to improve *Miscanthus* salt tolerance. Accession CN32 with the highest yield at a salt concentration below EC 2.5 dS/m was suggested to be planted in relatively low salt soils, while accessions CN14 and CN34 with relatively high salt tolerance and yield at EC 10 dS/m were recommended to be grown on strongly saline lands. For production biomass under moderately saline conditions, accession CN36 was the most promising candidate with highest yields at EC 6 dS/m. Combustion quality of the biomass under a salt stress level of EC 6 dS/m will likely not be adversely affected, as the genotypes did not accumulate ions in the shoot under these conditions.

This thesis has shown that *Miscanthus* germplasm has high genetic variation for salt tolerance. These results and conclusions provide insight in the influence of salt tolerance on biomass yield and quality, and revealed different traits and mechanisms underlying this tolerance. The salt tolerant genotypes can be directly used to produce biomass on marginal lands, or as starting materials in breeding programs to improve salt tolerance of *Miscanthus*.

Acknowledgements

Reaching the end of this incredible journey, I would like to express my sincerest gratitude to my supervisors, colleagues, families and friends that helped me in direct or indirect ways during my PhD process. Without your support and help, I could not have accomplished this journey. My apologies and thanks to all people I might have forgotten to mention in these acknowledgements.

First of all, I would like to give my thanks to my promotor Prof. Richard Visser giving me the opportunity to undertake my PhD in the Plant Breeding department of Wageningen University & Research. You are not only very inspiring but also encouraging. When I was writing my thesis at the most difficult moments, you encouraged me very positively and spent extra time editing my thesis. Your comments always made me see the bigger picture. Thank you for everything, Richard.

I deeply appreciate my supervisor and co-promotor Dr. Gerard van der Linden. Being a very clever person, you always point your considerations and different possibilities which generate very interesting discussions and hypothesis. I especially thank you for putting all the energy and efforts into helping me to finalise and submit my thesis before I left the Netherlands. We spent time together travelling to Dublin and Stuttgart for the conferences where you showed me how to collaborate with other groups and had humouros talks. Thanks for your company!

Dr. Oene Dolsma, it has been my honor to spend these years learning from you. I have learned so much knowledge and experience from you, and I still remember you said “breeding is a compromise”. After our discussions, I know how to continue the research in *Miscanthus* from different angles.

I would like to thank Dr. Luisa Trindade for her help in my project including pilot experiments for cell wall composition analysis, detailed protocols and important advices. We had a great collaboration throughout my PhD process and you also encouraged me to think deeper and try to see the bigger picture. Thank you for all the time you invested in guiding me!

I have to warmly thank my external supervisor, Dr. Xinyou Yin, for bringing perspective about what matters and for showing interest in my progress. For all this I am enormously thankful.

Throughout my PhD process, I am very grateful to my officemates: Dalia, Deniz, Dongli, Katharina, Mas, Michela, Myluska, Miguel, and Peter for showing valuable scientific knowledge, priceless friendships, memory and various cultures. I would like to sincerely thank each of you for support, patience and friendship during my time in Wageningen.

Since I have worked in the abiotic stress group, this group was like a family to me. I would like to acknowledge Baye, Biructawit, Christos, Clemens, Elly, Ernest, Engin, Hanneke, Long, Rafael, Sri, and Viviana. You helped me to adapt smoothly to the new environment and always spent time discussing. Best wishes from my heart for your careers and for happiness in your life! I appreciate the efforts of MSc students (Kim Sollie and Luc Bodinot) that have been involved in this project and helped me a lot. Both of you showed your best, and I spent a great time with you.

I would like to acknowledge some other people in the plant breeding department: Hanneke van der Schoot, thanks for all the help and advice in the lab and Unifarm. I really enjoyed working with you. Annemarie Dechesne, thanks for maintaining the IC system and clean working environment in the biochemical lab. Heleen Furrer, thank you for helping me with the cell wall composition analysis. Dr. Tim van der Weijde, I was invited to visit your field trial when I started my project. You also kindly provided the genetic map for me to perform the QTL analyses with that partly overlapped both of our individual projects. Dr. Andres Torres, thank you for sharing your passion and knowledge about cell wall biochemistry. Peter Dinh, you are one of the happiest and most positive persons, and it was always a pleasure to have a talk with you. Johan Bucher, you are one of the most optimistic and funniest persons and I was always full of joy after talking with you.

Many thanks to my Taiwanese friends throughout the years, A Chao Liang, Chaoyi Lin, Chia-Kai Kang, Chia-Yi Liu, Chih-Chung Wu, Chih-Chieh Chuang, Gary Lu, Han Tang, Hsuan Chen, Hsin-Ya Huang, I-Chiao Lee, Jenny Yeh, Jia-Yuh Lee (Eunice), Jie-Hong Huang (Red), Hieng-Ming Ting, Michelle Yeh, Meng-Yuan Jen, Wei-Shan Chen (MoMo), Terrence Lee, Tinyu Lai, Tzeyi Huang, Wei-Chan Hsieh, Ya-Fen Lin, Ying Huang, and Yu-Fang Chen, I want to express my appreciation for your company at Wageningen and for those great & funny times. My sincere thanks to Pengying Piena-Yang for initiating us kindly to celebrate and enjoy delicious food on holidays. Song-Bin Chang kindly helped me with my application to the PhD and had a tour at Wageningen University. I also would like to thank Chen Chang, Chien-Young Chu, Fan-Shin Liao, Hsueh-Shih Lin, Menq-Jiau Tseng, Sheng-Jung Ou, Shui-Ho Cheng, and Yu Sung for the inspiring talks and encouragement.

To my Chinese friends, Dandan Liu, Huayi Li, Xiaoxue Sun, Feng Zhu, Xiao Lin, Kaile Sun, Mengjing Sun, TingTing Xiao, Yiqian Fu, Yajun Wang, Xu Cheng, Xuan Xu, and Xuexue Shen. I am thankful for joyful moments and numerous scientific, social, different cultural discussions and delicious snacks during coffee breaks and lunch time.

Many thanks to the people of Unifarm: particularly Geurt Versteeg, Gerrit Huisman, Andre Maassen, and Rinie Verwoert. You arranged the hydroponic system and space for my experiments and gave me useful suggestions to run everything smoothly. Due to your help and care, I had enough healthy plants for every experiment.

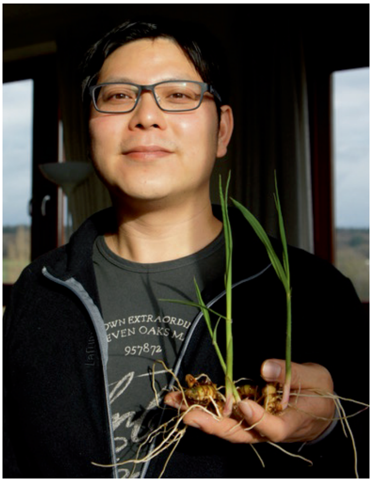
Many thanks to the secretaries of plant breeding: Janneke van Deursen, Nicole Trefflich and Letty Dijker. You are always a great support to everyone in the department, and you help us deal with the complexities of immigration issues, travel planning and expense reports.

To all the people in OPTIMISC: Andreas Kiesel, Iris Lewandowski, Olena Kalinina, Yasir Iqbal (Hohenheim University, DE), Heike Meyer, Kai-Uwe Schwarz (Schwarz, DE), Karl Müller-Sämann (ANNA, DE), Hilde Muyllen, Isabell Roldan-Ruiz, Peter Lootens, Simon Fonteyne (ILVO, BE), Chris Nunn, Iain Donnison, John-Clifton Brown, Kerrie Farrar, Laurie Huxley (Aberystwyth University, UK), Graham Harding, Michal Mos (Blankney Estates, UK), Ivan Tarakanov, Nikolay Khokhlov (Russian State Agrarian University, RU), Mensure Ozguven (Konya Food & Agriculture University, TR), Heinrich Schüle (DAZ, UA), and Qingguo Xi (Dongying Agricultural Institute, CN) - thank you all for the wonderful cooperation and the scientific discussions. Especially, John-Clifton Brown gave me useful suggestions to conduct my experiments. ILVO group supported the rhizomes as materials for my experiment, and Simon Fonteyne helped me dig rhizomes from the soil. Qingguo Xi conducted the field trial in China and supported the particular examples which extended bioenergy crops to the marginal lands. This valuable data not only generated chapter 5 but also indicated the high potential for production of *Miscanthus* on marginal lands.

I would like to express my heartfelt gratitude to my most loving parents, parents-in-law, brother, and brother- and sister-in-law in Taiwan for their full support and interest in my work. Thanks for everything!

A special acknowledgement goes to my wife, Chia-Chi, I cannot express in words my gratitude to you. You have always motivated me and stimulated me, and I would not be here without your continuous support. I wish you will pursue your goals without fear, and I will always be your backup.

About the author



Chang-Lin Chen was born on 23rd December 1979 in Kaohsiung City, Taiwan. He received his bachelor diploma in department of horticulture at National Chung-Hsing University, Taiwan in 2002. He continued to study at National Chung-Hsing University and got master's degree in graduate institute of molecular biology in 2004. He later worked as a research assistant in Taoyuan District Agricultural Improvement Station, Council of Agriculture Executive Yuan in Taiwan for four years. The major task was to collect the indigenous orchids in Taiwan and study genetic variation by morphological and molecular markers. Then, he joined the Technical Mission of the Republic of Taiwan to the Republic of Fiji as a specialist for 2 years. He managed the demonstration farm and supported the short-term training for local groups. He received studying abroad scholarship from Ministry of Education, Taiwanese government and started his PhD in plant breeding, Wageningen University & Research (WUR) in 2013, working on an EU 7 project focusing on identifying *Miscanthus* genotypes and mechanisms related to salt tolerance in this intersting bioenergy crop.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Chang-Lin Chen**
Date: **19 September 2018**
Group: **Plant Breeding**
University: **Wageningen University & Research**

1) Start-up phase	*date*
▶ **First presentation of your project**	
Salt tolerance in Miscanthus	10 Dec 10 2013
▶ **Writing or rewriting a project proposal**	
Salt tolerance in Miscanthus	16 Oct-16 Nov 2013
▶ **Writing a review or book chapter**	
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits **

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
EPS PhD student day, Leiden, the Netherlands	29 Nov 2013
EPS PhD student day 'Get2Gether', Soest, the Netherlands	29-30 Jan 2015
EPS PhD student day 'Get2Gether', Soest, the Netherlands	28-29 Jan 2016
EPS PhD student day 'Get2Gether', Soest, the Netherlands	09-10 Feb 2017
▶ **EPS theme symposia**	
EPS Theme 4 symposium ' Genome Biology', Wageningen, the Netherlands	16 Dec 2016
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Wageningen, the Netherlands	16 Jan 2017
EPS Theme 3 symposium 'Metabolism and Adaptation', Wageningen, the Netherlands	14 Mar 2017
▶ **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	11-12 Apr 2017
▶ **Seminars (series), workshops and symposia**	
Symposium: Omics Advances for Academia and Industry-Towards True Molecular Plant Breeding, Wageningen, the Netherlands	11 Dec 2014
Symposium: PBR Research Day, Renkum, the Netherlands	29 Sep 2015

Symposium: INTERWOVEN - How Science and Art Meet Belowground, Wageningen, the Netherlands	14 Dec 2015
Symposium: Kick-off Meeting of the Wageningen Plant Microbiome Network, Wageningen, the Netherlands	29 Jun 2016
Symposium: 1st WURomics Symposium, Wageningen, the Netherlands	15 Dec 2016
Invited seminar: George Coupland "Seasonal flowering in annual and perennial plants"	13 Jan 2015
Invited seminar: Yves van de Peer "The evolutionary significance of gene and genome duplications"	03 Feb 2015
Invited seminar: Siobhan Brady "Regulation of root morphogenesis in tomato species in the face of a changing environment"	9 Sep 2015
Invited seminar: Wilfred Vermerris "An introduction to sorghum breeding"	16 Nov 2015
Invited seminar: Wilfred Vermerris "Genetic and genomic resources for sorghum"	17 Nov 2015
Invited seminar: Sophie Nadot "Perianth evolution in Ranunculaceae: are petals ancestral in the family"	20 May 2016
Invited seminar: Hans Thordal-Christensen "Membrane trafficking in plant cells attacked by powdery mildew fungi"	12 Dec 2016
Invited seminar: Gerben van Ooijen "Clocks across taxa: Conserved cellular timekeeping mechanisms in plants, algae and other eukaryotes"	29 May 2017
Invited seminar: Antony van der Ent " Discovery of hyperaccumulator plants in Borneo and New Caledonia: multi-technique investigations"	11 Oct 2017
► **Seminar plus**	
► **International symposia and congresses**	
Plant Biology Europe FESPB/ESPO congress, Dublin, Ireland	24 Jun 2014
FP EU projects on bioenergy crops meeting, Dublin, Ireland	25-26 Jun 2014
Optimisc Annual Meeting 3, Dublin, Ireland	27 Jun 2014
Optimisc Annual Meeting 4, Wageningen, the Netherlands	20-21 Nov 2014
Perennial Biomass Crops for a Resource-Constrained World, Stuttgart, Germany	07-10 Sep 2015
Optimisc Annual Meeting 5, Stuttgart, Germany	11 Sep 2015
► **Presentations**	
Poster: Annual meeting 'Experimental Plant Sciences' - Evaluation of salt tolerance in Miscanthus using a hydroponic system	14-15 Apr 2014
Poster: Annual meeting 'Experimental Plant Sciences' - Genetic diversity of salt tolerance in Miscanthus	13-14 Apr 2015
Poster: Annual meeting 'Experimental Plant Sciences' - A comparison of the salt tolerance in Miscanthus between hydroponics and pots with different salt levels	11-12 Apr 2017
Talk: Optimisc Annual Meeting 3 - Evaluation of Miscanthus 70 genotypes	26 Jun 2014
Talk: Optimisc Annual Meeting 4 - Evaluation of salt tolerance in Miscanthus using a hydroponic system - Latest results	20 Nov 2014
Talk: Optimisc Annual Meeting 5 - Evaluation of salt tolerance in 12 Miscanthus genotypes	11 Sep 2015
Talk: Perennial Biomass Crops for a Resource-Constrained World - Genetic diversity of salt tolerance in Miscanthus	08 Sep 2015
Talk: Annual meeting 'Experimental Plant Sciences' - Exploring salt tolerance in Miscanthus: From hydroponics to pot experiments	12 Apr 2016

► **IAB interview**	
► **Excursions**	
EPS Company Visit Tomato world, Honselersdijk, the Netherlands	14 Oct 2016
Subtotal Scientific Exposure	*19.2 credits **
3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Basic Statistics, Wageningen, the Netherlands	20-27 May 2015
Microscopy and Spectroscopy in Food and Plant Science, Wageningen, the Netherlands	15-17 May 2017
9th International Utrecht PhD Summer School on Environmental Signaling in Plants, Utrecht, the Netherlands	28-30 Aug 2017
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	*3.4 credits **
4) Personal development	*date*
► **Skill training courses**	
Reviewing a Scientific Paper, Wageningen, the Netherlands	05 Mar 2015
The Essentials of Scientific Writing and Presenting, Wageningen, the Netherlands	09-22 Jun 2016
Data Management Planning, Wageningen, the Netherlands	06 Feb 2017
Research Ethics Seminar, Wageningen, the Netherlands	03 Apr 2017
Scientific Publishing, Wageningen, the Netherlands	13 Apr 2017
Posters and Pitching, Wageningen, the Netherlands	18 May 2017
Information Literacy including EndNote Introduction, Wageningen, the Netherlands	13-14 Jun 2017
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.1 credits **
TOTAL NUMBER OF CREDIT POINTS	***34.2 credits ****

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

The studies in this thesis received funding from the European Union consortium OPTIMISC (project ID 289159) under grant agreement n° 289159.

This thesis was financially supported by a scholarship from the Ministry of Education, Taiwan to the author.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design: Chia-Chi Chang and Chang-Lin Chen
Layout: Chia-Chi Chang
Printed by: ProefschriftMaken || DigiForce