



# Article Diversity in Phenological and Agronomic Traits of Miscanthus sinensis Collected in Korea and Eastern Asia

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Abstract: Four-year field experiments were conducted to investigate phenotypic traits associated with the biomass yield of 173 Miscanthus sinensis accessions collected from Korea and neighboring East Asian countries. Nine phenological and agronomic traits associated with biomass yield were assessed to investigate their phenotypic diversity and relationships with biomass yield as well as the latitudes of the *M. sinensis* accessions collection sites. Correlation analyses among phenological and agronomic traits, biomass yield, and collection site revealed that heading date, vegetative growth duration, leaf area, and stem growth traits (stem height, stem diameter, and stem dry weight) were closely related to biomass yield. The latitude of collection site exhibited a significant negative correlation with heading date, and heading date showed a significant positive correlation with biomass yield, indicating the high biomass potential of the accessions originating from lower latitude due to longer vegetative growth. The best biomass yield was mainly observed in M. sinensis accessions from the southern parts of Korea, such as Jeolla and Jeju provinces, with over 20 Mg DM  $ha^{-1}$ . Agronomic traits measured in the second year after planting also showed a high correlation with biomass yield measured in the fourth year after planting. In particular, vegetative growth duration, leaf area, stem diameter, and stem dry weight measured in the second year were significantly related to the fourth-year biomass yield. Therefore, these findings suggest that agronomic traits measured in the second year can be used for screening M. sinensis genetic resources and breeding lines with high biomass yield potential.

**Keywords:** agronomic trait; bioenergy crop; biomass yield; genetic diversity; *Miscanthus sinensis*; phenological trait

# 1. Introduction

*Miscanthus* species are exceptional among  $C_4$  grasses for their biomass productivity in temperate climates [1] and have been considered as a dedicated bioenergy crop due to their ideal characteristics, such as high biomass yield potential, broad environmental adaptation, low inputs after plant establishment, and high carbon sequestration [2]. For commercial biomass production from miscanthus, *Miscanthus* × *giganteus* has mainly been cultivated due to its high biomass yield and environmental adaptation to cold temperate regions. Many long-term field trials conducted in Europe revealed that the maximum biomass yield



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**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). potential of M. × *giganteus* was over 20 Mg dry matter (DM) ha<sup>-1</sup> year<sup>-1</sup> in Central Europe and 30–40 Mg DM ha<sup>-1</sup> year<sup>-1</sup> in Southern Europe [3,4].

However, because commercially cultivated miscanthus species are limited to M. × *giganteus* originating from a single clone, if any problems occur, such as biotic and abiotic stresses, they will become drastic and widespread in a short period of time due to a lack of genetic diversity [5,6]. Sole reliance on M. × *giganteus*, which has previously identified problems such as high risk of disease and pest infestation [7,8], could limit the expansion of *Miscanthus* commercialization as a bioenergy crop in the long term. Thus, sustainable bioenergy production of miscanthus requires breeding efforts to improve traits associated with biomass productivity and stress tolerance using more diverse genetic resources [9]. The study of genetic diversity in crop species has contributed to the conservation of genetic resources, broadening of the genetic bases, providing elite germplasm as a parental source, and practical applications in breeding programs [10]. To design a relevant breeding program, it is important to know the phenotypic variation and heritability of a trait [11]. Some researchers evaluated the genetic diversity of miscanthus using morphological traits and molecular markers [12–14] but limited information is available for genetic diversity and the relationship among the phenotypic traits related to biomass productivity.

As a parental species of  $M. \times giganteus$ , *Miscanthus sinensis* is broadly distributed from sub-tropic to sub-arctic regions in East Asia [2,5]. This broad geographical distribution implies high genetic diversity in *M. sinensis* collected from East Asia [15,16]. *Miscanthus sinensis* showed diverse phenotypic characteristics responding to environmental conditions such as latitude, altitude, and habitat [16–18]. However, many of these studies have some limitations, including a small number of miscanthus accessions [15–17,19], a lack of information on their origins [19], a small number of phenotypic characteristics assessed [16–19], and a small geographical scale where miscanthus was collected [16]. For a better understanding of miscanthus in its genetic diversity in association with biomass production and environmental adaptability, it is important to have diverse miscanthus accessions collected from broad geographical regions. A genetic diversity study with more diverse miscanthus accessions would result in a better ecological and physiological understanding and provide more diverse parental genetic resources (i.e., elite germplasm) for future breeding endeavors.

For cellulosic bioenergy crops, biomass productivity is one of the most important targets for breeding a new cultivar. Several studies investigated growth parameters to associate them with biomass yield, such as stem height, stem diameter, number of stems, flowering time, and autumn senescence rate [7,16–19]. Year and regional variations were also investigated in relation to biomass productivity [16–18]. These studies revealed that stem height, number of shoots, stem diameter, and flowering time are key phenotypic traits in determining miscanthus biomass. However, the number of traits and accessions tested was limited in many studies [15–19].

Environmental conditions such as latitude, rainfall, soil texture, and climate and genetic conditions such as phenological and agronomic traits are associated with miscanthus biomass yield [2,7,8,15,16,20–23]. Many studies have compared the phenotypic traits of miscanthus accessions, but the number of accessions is often limited, except in certain studies that assessed the phenotypic traits of a large number of accessions [17,21–23]. Robson et al. [23] assessed various phenotypic traits of 244 accessions collected from many different countries and analyzed their associations with each other, but there is a lack of information on the origins of the accessions. In the case of Zhao et al. [17]'s study, they tested 457 *M. sinensis* accessions collected in China but investigated only three phenotypic traits for 2 years, failing to show a clear relationship between phenotypic traits and biomass yield. Miscanthus biomass yield is affected by complex interactions between phenological and agronomic traits [21,24]; thus, more diverse phenotypic traits and their associations with biomass yield should be determined using a large number of miscanthus accessions.

Plant breeding is one of the main methods to improve productivity, quality, and other traits [11]. In the case of outcrossing an annual plant, breeding a new crop cultivar

from parental populations usually takes 7–10 years [25–28]. Miscanthus is a perennial outcrossing species, and even when propagated by rhizome, it requires 4 years to reach its maximum potential biomass yield after planting [2,7,18]. This indicates that a miscanthus breeding and yield evaluation may take four times longer than that of annual crops. Such a long period for phenotyping and screening is a major bottleneck in a miscanthus breeding program [21]. Thus, reducing the evaluation period to estimate miscanthus' performance in biomass production is key in reducing the breeding period. It can be hypothesized that earlier phenotypic traits assessed in the first or second year after a miscanthus planting may be related to later phenotypic traits closely associated with potential biomass yield can be effectively used for selection traits for miscanthus breeding, and thus, time and effort can be significantly saved.

Therefore, this study was conducted to determine the phenotypic diversity of *M. sinensis* by evaluating its phenotypic traits during the first four years after transplanting, to investigate the relationship between phenotypic traits and biomass yield, and to identify key traits at an early year after planting that can be utilized to estimate biomass yield potential of mature stands.

# 2. Materials and Methods

#### 2.1. Collection of M. sinensis Accessions

Rhizomes of *M. sinensis* were collected from various locations in Korea and the neighboring countries China, Japan, and Russia in autumn or early spring from 2008 to 2011. In total, 144 accessions were collected in Korea, 17 accessions in Northeastern China and Far Eastern Russia, and 12 accessions in Southern Japan (Figure 1). *Miscanthus*  $\times$  *giganteus* was used as a reference species. GPS information of collection sites was recorded using GPSMAP 60CS (Garmin Ltd., Lenexa, KS, USA).



Figure 1. Collection sites of Miscanthus sinensis accessions in Korea, China–Russia, and Japan.

# 2.2. Field Experiment

Field experiments were conducted from 2011 to 2014 at the Experimental Farm Station of Seoul National University located in Suwon (37°16'12.1" N, 126°59'27.5" E) and Yeoju (37°15′28.9″ N, 127°32′16.6″ E), Korea. Monthly temperature and precipitation were recorded at each site during the cultivation period (2011–2014) (Figure 2). As both sites are located in a similar latitude, no significant difference in temperature and precipitation was observed during the cultivation period. All collected miscanthus rhizomes were initially planted in plastic pots and placed in a glasshouse until they were transplanted in a field evaluation nursery. In April 2011, all the accessions were planted in the field located in Suwon and Yeoju at a density of 1 plant m<sup>-2</sup> accession<sup>-1</sup> by completely randomized planting with three replications (2 replications in Suwon and 1 replication in Yeoju). The soils field-tested from Suwon and Yeoju were a sandy loam with a pH of 5.60 and 4.90, electrical conductivity of 0.23 and 0.36 dS/m, total organic carbon of 1.26 and 2.60%, total nitrogen of 0.18 and 0.10%, available phosphorus (P) concentration of 100.33 and 134.84 mg kg<sup>-1</sup>, and soil cation exchange capacity (CEC) of 15.83 and 5.76 cmol kg $^{-1}$ , respectively. Nitrogen fertilizer (urea granule) was applied at 60 kg N ha<sup>-1</sup> year<sup>-1</sup> in early June, while other fertilizers such as phosphorus and potassium were not applied. Weed management was conducted manually and by sequential treatment of the following herbicides: S-metolachlor (Dual Gold<sup>®</sup>; Syngenta Korea, Korea) as a pre-emergence herbicide application at 750 g a.i. ha<sup>-1</sup> and dicamba (Banvel<sup>®</sup>; Sungbo Chemical Co., Ltd., Seoul, Korea) as a post-emergence herbicide application at 482 g a.i.  $ha^{-1}$  [29].



**Figure 2.** Monthly average air temperature (**A**,**B**) and precipitation (**C**,**D**) in Suwon and Yeoju during the cultivation period from 2011 to 2014 with the 10-year monthly average data (2005–2014).

#### 2.3. Assessment of Phenological and Agronomic Traits

From the first year after planting, 9 phenological and agronomic traits were assessed every year: first shoot emergence date, heading date, vegetative growth duration, flag leaf/second leaf/third leaf length and width for calculating leaf area, stem height, number of stems, stem dry weight, stem diameter, and biomass yield. The first shoot emergence date was measured when the first shoot emerged from the ground. The heading date was measured when the panicle emerged 1 cm from the tip of the stem. The vegetative growth duration was calculated as the difference in the first shoot emergence date and heading date. The *leaf length* and *width* of the second and third leaf from the flag leaf were measured when the panicle was fully emerged. *Leaf area* was calculated as follows [20]:

Leaf area 
$$(cm^2) = 0.74 \times leaf$$
 length  $\times$  leaf width (1)

At maturity in late November, stem height, the number of stems, and stem diameter were measured prior to harvest. Stem height was measured from the soil surface to the tip of the panicle. The number of stems was counted as the number of stems per plant in the space of 0.01 m<sup>2</sup> through the middle of the bunch contributing to the biomass yield (small stems were excluded according to Clifton-Brown and Lewandowski's method [18]). Stem diameter was determined at the midpoint between the second or the third basal nodes using Vernier calipers. All the stems with leaf sheaths were harvested from the whole bunch of the plant and oven-dried at 80 °C for 48 h, and the stem dry weight for the bunch was recorded. To estimate biomass yield (Mg ha<sup>-1</sup>), the dry biomass weight of the bunch was multiplied by the plant density of 13,300 plants ha<sup>-1</sup>.

# 2.4. Statistical Analysis

All the data were initially subjected to analysis of variance (ANOVA). Genetic variation and correlation analyses among phenological traits, agronomical traits, and biomass yield of *M. sinensis* accessions were conducted. Genetic variation among miscanthus accession was estimated using the mean data of agronomical traits recorded during the cultivation period. Correlation efficiency was calculated using Pearson's correlation coefficient. To examine the relationships among selected traits, linear regression analyses were conducted. All statistical analyses were performed using JMP 13 (SAS Institute Inc., Cary, NC, USA).

#### 3. Results and Discussion

## 3.1. Diversity in Phenological and Agronomic Traits

To evaluate the phenotypic diversity of *M. sinensis* accessions collected in wide geographic areas from a latitude of N 32° for the southernmost to N 43° for the northernmost in association with miscanthus biomass production, phenological and agronomic traits were measured in field conditions over four years. All the traits showed large variations, as summarized in Tables 1 and 2, Tables S1–S3 and Figure 3, which shows selected major phenological and agronomic traits. From the first year after planting to the fourth year after planting, agronomic traits related to biomass yield gradually increased every year (Table 2 and Tables S1–S3). Particularly, more notable increases in agronomic traits were observed between the second and the third year.

**Table 1.** Estimates of variance components for agronomic traits of miscanthus accessions.

		Estimates of Variance Components								
Source of Variation	Df	SE	HD	LA	РН	NS	SDW	SD	Yield	
Accession Year	172 3	12.24 *** 19.55 ***	389.15 *** 22.68 ***	3357.33 *** 6558.67 ***	635.95 *** 2098.49 ***	8.49 *** 6.96 ***	118.70 *** 37.29 ***	1.13 *** 1.64 ***	7.90 *** 19.72 ***	
Accession $\times$ year	516	37.69	26.27	2368.20	883.98	6.51	51.94	0.18	10.25	

SE; shoot emergence, HD; heading date, LA; leaf area, PH; plant height, NS; number of stems, SDW; stem dry weight, SD; stem diameter. \*\*\* indicates statistical significance at the 0.001 level.

		Korea ( <i>n</i> = 144)			Japan ( <i>n</i> = 12)			China–Russia ( <i>n</i> = 17)			M×
Traits	Unit	Range	Mean	Standard Error	Range	Mean	Standard Error	Range	Mean	Standard Error	giganteus
Latitude	N°	33.13-38.32			32.03-33.34			42.25-43.42			35.25 *
First shoot emergence date	Julian date	92.0-100.0	96.7	0.13	94.0-99.0	97.5	0.54	94.0–99.0	96.4	0.28	97.3
Heading date	Julian date	184.0-273.0	234.2	1.18	205.0-244.0	222.0	5.28	170.0-196.0	181.7	2.11	235.8
Leaf area **	cm <sup>2</sup>	138.0-681.0	286.0	7.39	180.0-434.0	280.2	24.70	115.0-217.0	150.5	7.11	454.3
Stem height	cm	82.0-363.0	237.2	3.80	150.0-341.0	277.1	15.31	116.0-219.0	178.2	7.34	380.9
Stem diameter	mm	3.24-9.38	6.00	0.10	4.82 - 7.98	6.78	0.27	2.62-5.20	4.09	0.17	9.0
No. of stem	Number 0.01 m <sup>-2</sup>	4.0-36.0	12.2	0.39	6.0-13.0	8.0	0.64	6.0-18.0	9.2	0.96	10.8
Stem dry weight	g stem <sup>-1</sup>	6.50-81.49	29.10	1.29	10.85-62.13	37.81	4.36	2.25-14.68	8.46	0.82	76.1
Biomass vield	$M\alpha$ ha <sup>-1</sup>	0 37-32 92	11 42	0.49	6 32_29 43	14 10	2 24	0 67-10 47	3 24	0.61	18 5

**Table 2.** Summary of phenological and agronomic traits of miscanthus accessions. Phenological and agronomic traits were assessed in the 4th year after miscanthus planting.

\* The latitude of M. × giganteus collection site was the latitude of Yokohama, where it was exported to Denmark in the 1930s [28]. \*\* Leaf area is the sum of the flag leaf area, the second leaf area, and the third leaf area.

Phenological traits are not biomass yield components but indirectly associated with plant biomass, which is mainly determined during vegetative growth duration, starting from the first shoot emergence and ending at the heading [22]. Therefore, variation in the first shoot emergence date and heading date is essential for evaluating phenotypic diversity in the biomass productivity of *M. sinensis*. In the fourth year after planting, the first shoot emergence date ranged from day 92 (Julian date) to day 100 with an average of day 96.7, showing a narrow variation of 8 days irrespective of collection site (Table 2, Figure 3A). In contrast, the first heading date ranged from day 170 to day 273 with an average of day 228.5, showing a much wider variation of 103 days and having a close association with collection site (Table 2, Figure 3B). The first heading date of Korean accessions collected at latitudes from N 33° to N 38° ranged from day 184 to 273, showing a maximum difference of 90 days between the earliest and the latest, suggesting high genetic diversity in heading and flowering time. Accessions from Southern Japan located between N 32° and N 33° showed day 205-244 for the first heading date. Accessions from Northeastern China and Far Eastern Russia located between N 42° and N 43° showed a much earlier first heading date, from day 170 to day 196, than those collected from lower latitudes, while accessions from Southern Korea and Southern Japan showed a very delayed first heading date (Table 2). These findings clearly demonstrate that the first heading date is closely associated with the latitude of the collection site. The narrow variation in the first shoot emergence date and the much wider variation in the first heading date result in wide variation in vegetative growth duration. A delayed vegetative growth stage may allow miscanthus to produce more biomass [22,23]. Previous studies also reported that the latitudinal origin of miscanthus accession affected their phenological traits, such as the first heading date and flowering date [7,30–32], resulting in variation in vegetative growth durations and biomass productivity. A similar result was also seen in switchgrass. Casler et al. [33] and Jensen et al. [32] revealed that biomass yields of switchgrass accessions were closely associated with the latitudes of their collection sites, which were also correlated with their phenological and agronomic traits.

The leaf area of all *M. sinensis* accessions gradually increased each year and showed wide variation depending on the collection site. In the fourth year, leaf areas of the second and the third leaves of *M. sinensis* accessions from Korea and Japan ranged from 138 to 681 cm<sup>2</sup> with an average of 286.0 cm<sup>2</sup> and from 180 to 434 cm<sup>2</sup> with an average of 280.2 cm<sup>2</sup>, respectively. Those from China and Russia ranged from 115 to 217 cm<sup>2</sup> with an average of 181.7 cm<sup>2</sup> (Table 2, Figure 3C). *Miscanthus sinensis* accessions from higher latitudes showed a smaller leaf area, while those from lower latitudes showed a larger leaf area, suggesting that leaf growth is negatively correlated with collection site latitude (Tables S4 and S5).



**Figure 3.** Distribution of phenological and agronomic traits of miscanthus accessions (first shoot emergence, **A**; heading date, **B**; leaf area, **C**; number of stem, **D**; stem height, **E**; stem diameter, **F**; stem dry weight, **G**; biomass yield, **H**). Box plots present mean (dashed line), median (line), interquartile range (boxes), and 5% to 95% percentile (whiskers) for each phenological and agronomic trait. All the traits were assessed in the 4th year after miscanthus planting.

In determining miscanthus biomass yield, stem traits such as number of stems and stem dry weight are important traits as a yield component. Number of stems showed wide ranges of variation but was not correlated with collection site (Tables S4 and S5). In contrast, other stem traits, such as stem height, stem thickness, and stem dry weight, showed correlations with collection site. We found that the higher the latitude is, the smaller are the stem height, stem thickness, and stem dry weight (Tables S4 and S5). Stem height of M. sinensis accessions from higher latitudes (China and Russia) ranged from 116 to 219 cm (average = 178.2 cm), while those from lower latitudes (Korea and Japan) ranged from 82 to 363 cm (average = 237.2 cm) and from 150 to 341 cm (average = 277.1 cm), respectively, with  $M. \times giganteus$  showing the tallest stem height of 380.9 cm (Table 2, Figure 3E). Stem diameter also showed broad ranges of distribution along the collection sites of miscanthus, ranging from 2.62 to 5.20 mm (average = 4.09 mm), 3.24 to 9.38 mm (average = 6.00 mm), and 4.82 to 7.98 mm (average = 6.78 mm) for China–Russia, Korea, and Japan, respectively (Table 2, Figure 3F), revealing that M. sinensis accessions from higher latitudes produce thinner stems than those from lower latitudes. Stem dry weight is determined by stem height and stem thickness.

The stem dry weight of miscanthus depends on individual stem growth, including stem height and stem thickness, and thus is an important trait in determining biomass yield [16,19,34,35]. Like stem height and stem thickness, stem dry weight also showed a clear relationship with origin latitude (Figure 4). *Miscanthus sinensis* accessions from lower latitudes showed greater stem dry weight than those from higher latitudes. In the fourth year, the stem dry weight of *M. sinensis* accessions from Korea and Japan ranged from 6.50 to 81.49 g stem<sup>-1</sup> (average = 29.10 g stem<sup>-1</sup>) and 10.85 to 62.13 g stem<sup>-1</sup> (average = 37.81 g stem<sup>-1</sup>), respectively Interestingly, the stem dry weight of those from China–Russia was much smaller, from 2.25 to 14.68 g stem<sup>-1</sup> (average = 8.46 g stem<sup>-1</sup>), than those from the lower latitudes of Korea and Japan, suggesting that *M. sinensis* accessions from lower latitudes have greater potential in stem growth and consequently biomass yield (Table 2, Figure 3G).

Biomass yields of *M. sinensis* accessions collected in Korea, Japan, and China–Russia ranged from 0.30 to 32.90 Mg ha<sup>-1</sup> (average = 11.40 Mg ha<sup>-1</sup>), 6.32 to 29.43 Mg ha<sup>-1</sup> (average = 14.10 Mg ha<sup>-1</sup>), and 0.67 to 10.47 Mg ha<sup>-1</sup> (average = 3.24 Mg ha<sup>-1</sup>), respectively, in the fourth year (Table 2, Figure 3H). Some of the *M. sinensis* accessions from Korea and Japan showed a greater biomass yield than *M.* × *giganteus*, of which the biomass yield was 18.50 Mg ha<sup>-1</sup>.

Overall, *M. sinensis* accessions from Korea and its neighboring countries showed high phenotypic diversity in their phenological and agronomic traits, except the first shoot emergence date (Tables 1 and 2, Tables S1–S3). Wide variation in heading date suggests that heading date determines the vegetative growth duration of miscanthus, resulting in wide variation in agronomic traits such as leaf area, stem height, stem diameter, and stem dry weight related to vegetative growth. Stem growth traits associated with stem and biomass also showed high phenotypic diversity, with a particular correlation between stem growth traits except the number of stem and the latitude of the collection site, as reported in previous studies [30,32]. The high diversity of our accessions in their phenotypic traits provides an opportunity to select an elite germplasm for a future breeding program.



**Figure 4.** Relationships between the latitude of miscanthus collection site and phenological and agronomic traits assessed in the fourth year after miscanthus planting (first shoot emergence date, **A**; heading date, **B**; leaf area, **C**; number of stem, **D**; stem height, **E**; stem diameter, **F**; stem dry weight, **G**; biomass yield, **H**).

#### 3.2. Relationships between Latitude of Collection Site and Phenotypic Traits

For a better understanding of how the latitude of the collection site is related to phenological and agronomic traits, correlation and linear regression analyses were conducted. Correlation analyses between traits assessed in the fourth year after planting and latitude revealed that most phenotypic traits were related to the latitude of the collection site, except the first shoot emergence date (Table S5). All phenological and agronomic traits except number of stems were negatively correlated with latitude. In particular, heading date, vegetative growth duration, leaf area, stem diameter, and stem dry weight showed greater negative correlations with *r* approximately -0.6 than other traits such as the first shoot emergence date, suggesting that accessions collected from lower latitudes may display greater value and possess greater potential in these traits. In contrast, the number of stems showed a positive correlation with latitude, indicating that the higher the latitude is, the greater is the number of stems. However, unlike our results, other studies reported that the number of stems was highly correlated with biomass yield and a good selection trait for estimating biomass yield of miscanthus [18,19].

Linear regression analyses between latitude and phenological and agronomic traits reconfirmed that heading is delayed with decreasing latitude (Figure 4B), resulting in increased vegetative growth duration, as the first shoot emergence date was not related to latitude (Figure 4A). Leaf area (Figure 4C), stem height (Figure 4E), stem diameter (Figure 4F), and stem dry weight (Figure 4G) also increased with decreasing latitude, resulting in increased biomass yield (Figure 4H). These findings suggest that accessions collected at lower latitudes have greater potential in biomass yield, although variation is wide among accessions collected in the same latitude. Studies conducted in Korea and China also showed similar results: accessions collected in lower latitudes produce greater biomass than those collected in higher latitudes [17,30,34]. Accessions collected at the higher latitudes of China–Russia started seedling emergence in the spring at a similar time to other accessions from lower latitudes (Figure 4A) but started heading much earlier, with no great variation, almost 40-50 days earlier than accessions from lower latitudes (Figure 4B), resulting in greatly shortened vegetative growth duration. Variation in phenological traits such as heading date and vegetative growth duration along latitude may affect the quantitative growth of miscanthus and lead to different performance in biomass components and biomass yield, although other factors may also be involved. Robson et al. [21–23] commented that the biomass yield of miscanthus was affected by complex associations among environmental factors, morphological traits, and phenological traits. A significant association between heading date and latitude of collection site suggests photoperiodic and thermal influences on heading and flowering date [7,30–32]. Similar cases were also found in other bioenergy grasses such as switchgrass, whose phenotypic traits were also affected by latitude of collection site, a result of latitudinal adaptation [33,36]. The significant relationship between latitude and agronomic traits (Figure 4) suggests that accessions collected from different geographical latitudes will provide more phenotypically diverse germplasms, which can be used for breeding new miscanthus varieties adapting to various latitudinal locations. However, prior to using them for breeding, it is necessary to investigate their genetic relationship in association with phenotypic variation.

#### 3.3. Relationships between Phenological and Agronomic Traits in Association with Biomass Yield

To evaluate relationships between phenological and agronomic traits in association with biomass yield, we conducted correlation and linear regression analyses between phenological traits, agronomic traits, and biomass yields of *M. sinensis* accessions measured in the second and fourth year after miscanthus planting (Figure 5 and Tables S4 and S5). Tables S4 and S5 show correlations between phenological traits, agronomic traits, and biomass yield assessed in the second year and fourth year after miscanthus planting, respectively. With the exception of the first emergence date and the number of stems, the other traits showed significant correlations with biomass yield consistently each year. In particular, stem dry weight (r = 0.619 \*\*\* and 0.467 \*\*\*), leaf area (r = 0.513 \*\*\* and

0.406 \*\*), stem height (r = 0.467 \*\*\* and 0.373 \*\*), heading date (r = 0.378 \*\* and 0.373 \*\*), vegetative growth duration (r = 0.441 \*\* and 0.367 \*\*), and stem diameter (r = 0.260 \*\* and 0.393 \*\*) were significantly correlated with biomass yield assessed in the second and fourth year after miscanthus planting, respectively. These results suggest that these traits are important for biomass formation in miscanthus. In determining miscanthus biomass yield, the number of stems and stem dry weight are considered important traits as yield components [16,18,30,37]. However, in our study, the number of stems showed no significant correlation with other agronomic traits and biomass yield. This difference might be due to differences in counting the number of stems. We counted the number of stems in the space of  $0.01 \text{ m}^2$  through the middle of the bunch, while the other studies measured the total number of stems. In contrast, stem dry weight showed a strong correlation with the main phenological and agronomic traits (Figure 5). In the second year after miscanthus planting, heading date, vegetative growth duration, leaf area, and stem diameter were strongly correlated with stem dry weight, which was strongly correlated with biomass yield (r = 0.619 \*\*\*) but not much correlated with number of stems (Figure 5A). These correlations were maintained similarly in the fourth year after miscanthus planting (Figure 5B), suggesting that six phenotypic traits (except number of stem) are key traits determining miscanthus biomass yield.

Linear regression analyses revealed that stem diameter is the most significant contributor to stem dry weight ( $r^2 = 0.830$ ), followed by leaf area ( $r^2 = 0.613$ ), heading ( $r^2 = 0.321$ ), vegetative growth duration ( $r^2 = 0.305$ ), and stem height ( $r^2 = 0.279$ ) (Figure 6). Our findings that leaf area and stem dry weight were most strongly related to biomass suggest that leaf area plays an important role in stem growth, particularly stem diameter, and biomass accumulation. Previous studies reported that vegetative growth durations, stem height, stem diameter, and stem dry weight were strongly correlated with biomass yield potential [8,11,16–23,30,34,38]. However, the linear relationships between biomass yield and phenotypic traits were not strongly supported by the regression coefficients (Figure 6C,F,I,L,O). It can be assumed that biomass yield is affected by complex interactions of environmental factors and various phenotypic traits. Clifton-Brown et al. [7] and Robson et al. [21] reported that miscanthus biomass yield is a combined result of complex associations with environmental factors and phenotypic traits. Although our results suggested that miscanthus biomass is more attributed to leaf area, stem diameter, and stem weight than the other traits, these findings do not mean that the other traits should not be taken into account. Our results imply that the miscanthus accessions tested in this study are genetically very diverse.

## 3.4. Early Traits Determining Biomass Yield Potential of Miscanthus

Producing high biomass is the main goal for miscanthus cultivation. Moreover, miscanthus requires at least three to four years after planting for commercial biomass production, and phenotyping of agronomic traits associated with biomass yield requires more than three years in the field, costing significant time, effort, labor, and money. Additionally, breeding miscanthus has faced several obstacles, such as a lack of available germplasm and complex interactions of phenotypic traits in determining biomass. Thus, finding key traits determining miscanthus biomass yield is important in miscanthus breeding. Many studies have focused on those traits that are associated with biomass yield productivity [16,17,19,38], but they investigated relationships between traits and biomass yields assessed in the same year. However, if certain traits measured in the early stage of establishment are related to biomass yield potential, which can usually only be assessed in the fourth year after miscanthus planting in the field, we can significantly shorten the period for screening candidate lines.



**Figure 5.** Schematic representation of the correlation between phenological trait (heading date), main agronomic traits, and biomass yield assessed in the 2nd (**A**) and 4th (**B**) year after miscanthus planting. \*, \*\*, and \*\*\* indicate statistical significance at 0.05, 0.01, and 0.001 levels, respectively.

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**Figure 6.** Relationships between phenological and agronomic traits and main yield components number of stem (**A**,**D**,**G**,**J**,**M**), stem dry weight (**B**,**E**,**H**,**K**,**N**), and biomass yield (**C**,**F**,**I**,**L**,**O**) assessed in the 4th year after miscanthus planting.

To examine early phenotypic traits that are closely associated with the fourth-year biomass yield, we compared phenotypic traits assessed in the second year and the fourth year after miscanthus planting. Significant correlations exist between the same traits assessed in the second and fourth year after planting: heading date (r = 0.943 \*\*\*), vegetative growth duration ( $r = 0.909^{***}$ ), leaf area ( $r = 0.470^{***}$ ), stem height ( $r = 0.270^{**}$ ), stem diameter (r = 0.869 \*\*\*), number of stem (r = 0.995 \*\*\*), and stem dry weight (r = 0.786 \*\*\*) (Table 3). Correlation analyses between the main phenotypic traits assessed in the second year and biomass yield assessed in the fourth year showed significant correlations, except number of stems (Table 3, Figure 7). As expected, the second-year biomass yield showed the greatest positive correlation with the fourth-year biomass yield (r = 0.785 \*\*\*). Among individual phenotypic traits, stem dry weight showed the next greatest positive correlation with the fourth-year biomass yield (r = 0.525 \*\*\*), suggesting that stem dry weight is one of the most significant contributors determining miscanthus biomass yield. Other traits such as vegetative growth duration, leaf area, stem height, and stem diameter showed similar correlations with the fourth-year biomass yield (Figure 7). Anzoua et al. [16] investigated the relationship between agronomic traits and biomass yield potential and revealed that biomass production was strongly correlated with stem height and leaf length. The other studies reported that stem diameter, stem height, and heading date showed significant correlations with biomass yield [19,34,35]. Clifton-Brown and Lewandowski [18] suggested that at least 2 years of field trials are required to predict the biomass yield of miscanthus and that first-year data are insufficient to predict biomass yield. These previous studies investigated the relationships between phenotypic traits and biomass yield assessed in the same year, while our study investigated the relationships between the second-year phenotypic traits and the fourth-year biomass yield. Our results suggest that potential biomass yield can be predicted in an early year of miscanthus cultivation—for example, the second year after planting. The early traits showing high correlations with the fourthyear biomass yield may be utilized as selection traits for screening breeding lines with high biomass yield potential in a miscanthus breeding program, saving time and effort in phenotyping and screening.



**Figure 7.** Schematic representation of the correlation between phenological (heading date) and main agronomic traits assessed in the 2nd year after miscanthus planting, and biomass yield assessed in the 4th year after planting. \*, \*\*, and \*\*\* indicate statistical significance at 0.05, 0.01, and 0.001 levels, respectively.

Traits in the 2nd	Traits in the 4th Year after Planting										
Year after Planting	Heading Date	Vegetative Growth Duration	Leaf Area	Stem Height	Stem Diameter	Number of Stem	Stem Dry Weight	<b>Biomass Yield</b>			
Heading date	0.943 ***	0.943 ***	0.595 ***	0.346 **	0.610 ***	-0.089	0.607 ***	0.366 **			
Vegetative growth duration	0.907 ***	0.909 **	0.551 ***	0.358 **	0.528 ***	0.100	0.519 ***	0.381 **			
Leaf area	0.434 **	0.446 **	0.470 ***	0.187 *	0.406 **	0.291 **	0.368 **	0.404 **			
Stem height	0.150 *	0.160 *	0.183 *	0.270 **	0.131 *	0.574 ***	0.038	0.338 **			
Stem diameter	0.600 ***	0.586 ***	0.735 ***	0.559 ***	0.869 ***	-0.267 **	0.808 ***	0.360 **			
Number of stem	-0.037	-0.020	-0.202 **	-0.171 *	-0.310 **	0.995 ***	-0.322 **	0.112			
Stem dry weight	0.567 ***	0.560 ***	0.668 ***	0.413 **	0.725 ***	-0.113	0.786 ***	0.525 ***			
Biomass yield	0.375 **	0.372 **	0.304 **	0.338 **	0.281 **	0.259 **	0.337 **	0.785 ***			

**Table 3.** Correlation coefficients between phenological and agronomic traits assessed in the 2nd year and the 4th year after miscanthus planting.

\*, \*\*, and \*\*\* indicate statistical significance at the 0.05, 0.01, and 0.001 levels, respectively.

# 4. Conclusions

In this study, we found that our miscanthus accessions showed high diversity in phenological and agronomic traits, and these traits were strongly correlated with latitude of collection site. The best biomass yield was mainly observed in *M. sinensis* accessions from the southern parts of Korea, such as Jeolla and Jeju provinces, with over 20 Mg DM ha<sup>-1</sup>, which may be mainly attributed to longer vegetative growth. Our study clearly demonstrated that latitude of collection site affects phenological traits, particularly heading date, and consequently vegetative growth, which determine the biomass yield of miscanthus in a combined manner. Relationship analyses between earlier traits assessed in the second year and later traits assessed in the fourth year suggest that earlier traits may be useful parameters to estimate relevant parameters and potential biomass yield in perennial crops including miscanthus. Among traits assessed in the second year after miscanthus planting, leaf area of the second and third leaves from the flag leaf was closely associated with stem diameter and stem dry weight. Stem diameter and stem dry weight assessed in the second year were highly correlated with those traits and biomass assessed in the fourth year, suggesting that leaf area, stem diameter, and stem dry weight are key traits in estimating potential biomass yield. These traits can be used as a target trait for screening miscanthus breeding lines with high biomass yield potential. Considering our results, it can be concluded that the selected traits, such as vegetative growth duration, leaf area, stem diameter, and stem dry weight, observed in the second year after miscanthus planting may help us to predict the potential biomass yield of miscanthus and screen candidate lines with our target traits in a miscanthus breeding program. It is necessary to validate our findings and the phenotypic traits proposed in other independent field studies using different plant materials.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10 .3390/agronomy11050900/s1, Table S1: Summary of phenological and agronomic traits of miscanthus accessions. Phenological and agronomic traits were assessed in the 1st year after miscanthus planting, Table S2: Summary of phenological and agronomic traits of miscanthus accessions. Phenological and agronomic traits were assessed in the 2nd year after miscanthus planting, Table S3: Summary of phenological and agronomic traits of miscanthus accessions. Phenological and agronomic traits were assessed in the 3rd year after miscanthus planting, Table S4: Correlation coefficients between latitude of miscanthus collection site, phenological traits, agronomic traits and biomass yield assessed in the 2nd year after miscanthus planting, Table S5: Correlation coefficients between latitude of miscanthus collection site, phenological traits, agronomic traits and biomass yield assessed in the 2nd year after miscanthus planting, Table S5: Correlation coefficients between latitude of miscanthus collection site, phenological traits, agronomic traits and biomass yield assessed in the 2nd year after miscanthus planting, Table S5: Correlation coefficients between latitude of miscanthus collection site, phenological traits, agronomic traits and biomass yield assessed in the 4th year after miscanthus planting.

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