“Two-steps” seed-derived plugs as an effective propagation method for the establishment of Miscanthus in saline–alkaline soil

Cheng Zheng1 | Shuai Xue2,3 | Liang Xiao2,3 | Yasir Iqbal2 | Guorong Sun4 | Meijuan Duan1 | Zili Yi2,3

Abstract

Miscanthus, a perennial rhizomatous C4 grass, has emerged as a promising lignocellulosic crop. To avoid competition with food crops, marginal lands especially saline–alkaline soil are being recommended to grow Miscanthus across China. However, the challenges such as high cost and low reproduction coefficient associated with the propagation methods are impeding its large-scale plantation in saline–alkaline soil. In this study, a “two-steps” propagation method was designed, which was comprised of two phases: (1) seedling cultivation and (2) seedling strengthening. The parameters (substrate composition and plug size) for this method were determined. The optimal substrate composition of the seedling bed for M. sinensis, M. lutarioriparius, and their interspecific hybrids was peat and perlite at a ratio of 3:2, 3:1, and 3:3 by volume, respectively. The seedlings were transplanted into the plugs to deliver robust plantlets. The most appropriate plug size for M. sinensis, M. lutarioriparius, and their interspecific hybrids was 50 plugs (50 cm3/plug), 72 plugs (40 cm3/plug), and 72 plugs (40 cm3/plug), respectively. Three propagation methods, that is, direct sowing of seeds, rhizomes plantation, and “two-steps” seed-derived plugs for M. lutarioriparius were compared in saline–alkaline soil over 3 years (2014, 2015, and 2016). The establishment rate of the seeds directly planted was poor (zero). The performance (e.g., biomass yield) of the plants derived from the “two-steps” seed-derived plugs was better than that of the plants derived from rhizomes directly planted. Therefore, the “two-steps” seed-derived plugs can be regarded as an effective propagation method to upscale Miscanthus cultivation in saline–alkaline soil.

KEYWORDS
bioenergy crop, Miscanthus, plug seedlings, rhizomes directly planted, saline–alkaline soil, seed propagation
1 | INTRODUCTION

*Miscanthus*, a C4 perennial grass, has been widely accepted as a promising eco-industrial crop, because of its high resource use efficiency and wider adaptability to diverse environmental conditions (Harvolk et al., 2014; Jihoon & Dossoon, 2012; Yan et al., 2011). However, to avoid competition with food crops, marginal lands especially saline–alkaline soil must be exploited to grow industrial crops for the populated countries like China (Gopalakrishnan et al., 2011; Lewandowski et al., 2003; Zheng et al., 2019). A case study in China has estimated the potential of marginal lands to produce *Miscanthus* biomass, which is $3.85 \times 10^7$ t/y of which contribution of saline–alkaline soil is 28.49% (Xue et al., 2016). Estimates from the coastal flatland and inland plain, for example, the Huang-huai-hai Plain, the Northeast Songnen Plain, and the Yellow River Delta. The gentle terrain features and continuous large-scale distribution of the saline-alkaline land make it easy to achieve mechanized production. It suggests that the maximization of the saline-alkaline land potential could be the focal point for the development of the *Miscanthus* production industry in China.

Currently, lack of saline-alkaline tolerant varieties and inefficient establishment techniques are being considered as major challenges to expand *Miscanthus* cultivation (Scordia et al., 2015; Xue et al., 2015). Shortage of the varieties can be addressed by directly screening or domesticate saline-alkaline tolerant genotypes from natural germplasm sources as well as artificial hybridization. Current propagation methods have number of limitations such as low multiplication ratio of rhizomes and high production cost, which is subsequently limiting the upsaling of *Miscanthus* (Mangold et al., 2017; Xue et al., 2015). Seed propagation, with the highest multiplication ratio (approximately 1:1200) and the lowest establishment cost can contribute toward overcoming the aforementioned challenges (Xue et al., 2015). That is why it is being considered as an efficient method to expand the industrial applications of *Miscanthus* (Boersma & Heaton, 2014; Xue et al., 2013, 2015). However, currently the extremely low field emergence rate (<10%) and the poor over-wintering performance are the major problems associated with the direct seed sowing (Clifton-Brown et al., 2016). It drops even further in case of direct seed sowing of *Miscanthus* under marginal conditions such as saline–alkaline soil (data no shown). Therefore, optimizing the *Miscanthus* seed propagation method for saline–alkaline soil is essential for the development of bio-based industry in China.

Seed-derived plugs, which enhance the seedling adaptability to the environment, have been used commercially in the production of vegetables and flowers. This method has the advantage over conventional direct seed sowing mainly through increasing crop uniformity and promoting efficient growth. *Miscanthus* with comparatively small-sized seeds does not fit into currently available conventional direct seed sowing technology. Furthermore, improving seed size or quality of seed through breeding requires longer time period. Therefore, focus should be on identification of optimal seed propagation technique. Seed-derived plugs can be a viable option which must be adjusted and adapted to set up an efficient propagation method to upscale *Miscanthus* cultivation. However, early attempts show that the current empirical seed-derived plug propagation has a high blind planting (i.e., no seedling survived in the plug rate ranging from 20% to 40% for *Miscanthus*, which renders mechanized transplanting in the field (Clifton-Brown et al., 2016). Therefore, to optimize the seed-derived plugs propagation technique, more studies are required to achieve healthy seedlings from the seeds so that the success of generating seedlings from seeds is improved. However, these studies generally require a relatively long period for improving seed by breeding. To speed up the solution of the problem, the method of seed propagation was considered. In this study, a method comprised of two steps was set up to counter the issue of high blind placement rates. Two steps are consisted of: (1) seedling cultivation which involves sowing the seeds into the seed generation bed to produce seedlings; (2) seedling strengthening through transplanting the seedlings into the seedling–raising bed to achieve robust seedlings (Figure 1). Currently, to set up an efficient “two-steps” seeds propagation method, it is important to identify the optimal substrate composition and plug size. There are number of evidences from literature suggesting that the germination of seeds and the growth of seedlings were significantly influenced by the substrate composition of the seedbed (Leroy et al., 2017; Meng et al., 2017; Otilia et al., 2015). In addition, the utilization of larger plug sizes generally generates strong plantlets, but increasing the cost. Therefore, further studies are needed to balance the cost and the plantlets performance (Romero-Munar et al., 2018). Thus, based on personal communications with the *Miscanthus* plantlet production companies and our own experience, peat, perlite, and spent mushroom substrate were selected as substrate components to prepare seed germination bed for *Miscanthus*. However, their optimal composition ratios are still unclear. In addition, four plug sizes (32, 50, 72, and 128 plug tray) with the most widely and commercially used for seedling production were screened for the most suitable size for *Miscanthus* nursery.

Therefore, this propagation method is developed through designing experiments, which involves: (1) determining the optimized substrate composition and cell size for the seedling emergence and growth; (2) defining the genotype-substrate/plug size-specific relationship; and (3) evaluating the performance of “two-steps” seed-derived
seedlings transplanted in the saline–alkaline soil. Two questions (shown in Figure 1) will be answered for the first aim and one for the third aim.

2 | MATERIALS AND METHODS

2.1 | Experiment I: Screening the substrate composition of the seedbed

Based on the outstanding performance on the saline-alkaline land in the Yellow River Delta area, three genotypes, including *Miscanthus sinensis*, *Miscanthus lutarioriparius*, and one interspecies hybrid (*M. lutarioriparius* (♂) × *M. sinensis* (♀)), were selected as the research material (Zheng et al., 2019). Seeds of *M. sinensis* were open pollinated and collected from the experimental stations at Liuyang (E113°04′E, N28°10′N). The plantation area was surrounded by *Arundo donax* and *Pennisetum alopecuroides* (L.) Spreng. *M. lutaria-riparius* seeds were collected from a natural population at Yuanjiang (112°19′E, 29°03′N). Seeds of the hybrid were collected from the open-pollinated hybrid plants, which were planted at the Hunan Agricultural University campus experimental station (113°53′E, 28°17′N) in Changsha with many other hybrids surrounding them. All the seeds were collected at the end of the 2013 growing season and then threshed and placed in a room temperature dryer. The basic quantitative properties of the seeds are listed in Table S1.

*Miscanthus* seed germination bed. There are nine composition treatments included with their details shown in Table S2. All the factorial treatments (nine compositions and three genotypes) with three replicates were arranged in a randomized complete block design. In May 2014, seeds of all the treatments and replicates were directly sown in 81 trays (50 × 25×8 cm). Fifty seeds were sown evenly in each tray at a depth of approximately 0.3 cm.

All the used substrates had a water content of 100%, before seed sowing. After sowing the seeds, all the trays were placed in the growth chamber, where growth conditions were maintained with temperature 28.0°C (during day) and 25.0°C (during night), air relative humidity ≥75%, and a 16/8 h light/dark period under the randomized complete block design. The trays were sprayed with 30 ml of water each day until the end of the experiment.

When the germination rate (GR) was constant (in this case, 35 days after seeds sowing), the final GR and germination index (GI) were calculated. The final GR was defined as the percentage of seedlings that germinated from the seeds sown initially as shown in Equations (1) and (2):

\[
\text{Germination rate} = \frac{\text{Final germinated seeds}}{50} \times 100\% , \quad (1)
\]

\[
\text{Germination index} = \sum_{t=1}^{35} \left( \frac{E_t}{D_t} \right) , \quad (2)
\]

where \(E_t\) was the number of seedlings per tray at the \(t\) th days, and \(D_t\) was the \(t\) th day after sowing the seeds. After seeds sown for 35 days, the following traits of the seedlings were measured, to get the seedling height, root length (RL), root average diameter, root surface area (RSA), and root volume. All these traits were measured based on nine randomly selected seedlings in each tray. RL, average root diameter (RD), RSA, and root

![Diagram of seed-based "two-steps" propagation method](image_url)
volume were measured using calibrated WinRhizo image analysis software (Regent Instruments).

2.2 Experiment II: Screening the optimal plug size for robust seedlings

An optimal plug size to produce strong plantlets is also required to balance the economic input and the plantlet quality. An experiment comprised of four plug size treatments and one control treatment (seedlings were directly planted in tray without plug) was designed here. The four plug size treatments were 32 plug size per tray (140 cm³/plug), 50 plug size per tray (50 cm³/plug), 72 plug size per tray (40 cm³/plug), and 128 plug size per tray (17 cm³/plug). All the factorial treatments (five plug sizes and three genotypes) with three replicates were arranged in a randomized complete block design. All the seedlings used in this Experiment II had three leaves and a uniform plant height (approximately 6.0 cm tall) were randomly selected from Experiment I during June 2014. The selected seedlings were transplanted into different treatments. To eliminate the effect of the substrates on the growth of seedling, all the substrates were prepared using peat and perlite at ratio of 1:1 by volume. All the trays in the growth chamber were placed under the same growth conditions as mentioned above. The trays were watered with 50 ml of water each day until the end of the experiment.

The experiment was terminated after the seedlings grown for 30 days in the growth chamber. Then, data were collected, including seedling height, stem diameter, RL, and total plant dry weight. The plant was divided into underground and aboveground parts, which were dried at 80°C until constant dry weight. The plant was divided into underground and aboveground parts, which were dried at 80°C until constant dry weight. The healthy seedling index (HI) was calculated using the following equation:

\[ HI = \left( \frac{\text{root dry weight}}{\text{aboveground dry weight}} \right) + \left( \frac{\text{stem diameter}}{\text{plant height}} \right) \times \text{total plant dry weight}. \]

2.3 Field performance parameters

A field trial was set up to examine the field performance of the plants developed through the “two-steps” seed propagation method on the saline-alkali land and further compared with those from the rhizome-propagated plants. The field trial was conducted at BinZhou, Shandong Province (38°37′N, 118°07′E, 3.8 m a.s.l.). The trial field was characterized by coastal saline-alkali soil. Physical and chemical properties (0–20 cm) are presented in Table 1. The annual maximum, minimum temperature, and rainfall distribution of the trial site are shown in Figure 2. The cumulative rainfall was 431.9 mm in 2014, 628 mm in 2015, and 439.6 mm in 2016. The average temperature during 2014, 2015, and 2016 was 14.4°C, 14.1°C, and 14.2°C, respectively.

The field trial involved three propagation treatments (the “two-steps” seed propagation, rhizome directly planted, and seed directly planted) and three replications in a randomized complete block design. The plot size was 3 by 16 m. Based on the results of experiment I and experiment II, plantlets derived by the above-confirmed optimal propagation parameters (peat:perlite = 3:1 by volume for seed germination bed; 72 plugs/tray for robust seedlings) were used in this trial. In June 2014, strong plantlets were manually transplanted into the field plot. Simultaneously, the rhizome cuttings of approximately 200 g, which were harvested at the same site as the seeds on November 12, 2013 and stored under the conditions of 5°C and 75% humidity, were also planted manually. The plantlets and rhizomes were planted with 1 m planting space and 1 m row spacing. The seeds were evenly sown with a depth of <0.5 cm and seeding density of 100 seeds per square meter. To ensure good soil conditions for the growth of the plants, the field was harrowed before planting. The plots were irrigated once after planting, and no irrigation or fertilization was applied during the experimental period of 2014–2016. To control weeds, during the first year of plantation (2014), manual weeding was carried out twice (on July 20 and August 30) for all plots.

The survival rate was determined by the ratio of the survived plants that counted in November 2014 to the total number of seedlings or rhizomes initially planted. The overwintering survival rate was calculated during spring of 2015 as the ratio of successfully sprouted plants to the total number of seedlings or rhizomes initially planted. The final establishment rate was defined as the ratio of the number of plants at the end of the 2016 growing season to the total number of seedlings and rhizomes planted in 2014.

Over the whole experimental time period (2014–2016), six morphological traits, which include the single stem weight (with leaf), plant height, tiller diameter, tiller number, biomass yield, and stem/leaf ratio, were measured annually at the end of each growing season. Five plots were randomly selected and

| Items                               | Value  |
|-------------------------------------|--------|
| pH                                  | 9.0    |
| Organic matter (g/kg)               | 13.54  |
| Total phosphorus (g/kg)             | 0.71   |
| Total nitrogen (g/kg)               | 24.09  |
| Total potassium (g/kg)              | 1.34   |
| Available phosphorus (mg/kg)        | 48.64  |
| Available nitrogen (mg/kg)          | 6.10   |
| Available potassium (mg/kg)         | 338.64 |
| Electrical conductivity (EC) (dS/m) | 6.55   |
1 × 1 m quadrats per plot were harvested leaving stubble height of 5 cm. Biomass samples were weighted. A single stem (with leaf) per plot was randomly selected from the harvested biomass, then dried at 80°C for 7 days, and weighed to calculate biomass yield. Biomass yield = fresh biomass × (dry weight of tiller/fresh weight of tiller). Fresh biomass is the fresh biomass yield of 1 × 1 m quadrat. Tiller biomass is the dry weight of a single stem. Fresh weight of tiller is the fresh weight of a single stem. The agronomic traits of the plug seedling-derived plants and rhizome-derived plants were investigated. The plant height was measured from the ground to the top of the tallest shoot. The tiller numbers per square meter were counted. The tiller diameter was measured at 10 cm from the base of the stem. Stem/leaf = stem dry weight/leaf dry weight.

### 2.4 Data analysis

The principle component analysis (PCA) and membership function (MF) were adopted to evaluate the effect of the substrates on the germination and growth of seedlings. Because substrate treatments have impacts on the traits of seed germination and seedling growth, correlation coefficients were calculated among GR, GI, SH RL, RD, RSA, and RV. The PCA, as a statistical method, is used to evaluate the performance of germination and seedling under specific substrate.

Contribution of the trait variables to define the effect of substrate on germination and seedling of Miscanthus was shown in Figure S1. Results of the PCA analysis show that the first three principle components (PC1, PC2, and PC3) have explained 73.00% of all the contributions of variables. Among PC1, RSA had the highest contribution (0.967) to screen substrate components, followed by RL (0.842). GR, GI, RSA, RL, and RD were used as key traits to screen the optimal substrate composition.

The selected traits were used by MF analysis to screen the most optimal substrate treatment. The MF was adopted to evaluate the effect of the substrates on the germination and growth of seedlings. The MF of a fuzzy set is a generalization of the indicator function in classical sets, which represents the degree of truth as an extension of valuation. The formula is shown as Equations (3) and (4):

\[ F_i = \frac{1}{n} \sum_{j} F_{ij}, \]  
\[ F_{ij} = \frac{(X_{ij} - X_{ij\text{min}})}{(X_{ij\text{max}} - X_{ij\text{min}})}. \]
where $F_i$ is the average value of the MF of all the traits ($n = 6$: GR, GI, seedling height, RL, root average diameter, RSA, and root volume) for the genotype ($i$). $F_j$ is the MF value of the trait ($j$) for the treatment ($i$) for a genotype; $X_{ij}$ is the measured value of the trait ($j$) for the treatment ($i$) for a certain genotype; $X_{j\text{max}}$ is the maximum value of the trait ($j$); $X_{j\text{min}}$ is the minimum value of the trait ($j$).

General analyses of variance were performed to determine the significance of genotype, treatment, and interaction sources of the variation. For growth-related traits, ANOVAs were performed following the completely randomized block design of the experiment. Genotype and treatment were set as the fixed factor.

$$Y_{ijk} = u + G_{ij} + T_k + GT_{ijk} + e_{ijk}, \quad (5)$$

where $Y_{ijk}$ is the response variable; $u$ is the grand mean; $G_{ij}$ is the genotype effect; $T_k$ is the treatment effect; $GT_{ijk}$ is the interaction term between genotype and treatment, and $e_{ijk}$ is the residual error. In terms of the field trial, all the agronomic traits were subjected to a two-way ANOVA using repeated measurements in time. The measurements included the performance year as the within subject and the propagation methods as the between subject. All the analyses were conducted in SPSS 19.0 (SPSS Inc.). The difference between the treatment means was compared using Duncan’s multiple range test.

3 | RESULTS

3.1 | Optimal substrate components for Miscanthus

We applied the MF to estimate the most suitable substrate for the three Miscanthus genotypes (Figure 3). The MF value of the GR and GI at the specific germination stage, RL, RD, and RSA at the seedling stage (Figure S1) were calculated. The results showed that the subordinate function values of T1 (peat:perlite = 1:1; 0.98), T2 (peat:perlite = 3:2; 0.92), and T3 (peat:perlite = 3:1; 0.84) were the highest for M. lutariairoparius, M. sinensis, and the hybrids, respectively.

The GR of seed, RL, RD, and RSA of seedling were 73.33%, 13.9 mm, 0.23 mm, and 0.126 cm$^2$, respectively, for T1-treated M. lutariairoparius seed and seedling. In addition, it has the highest the subordination values in comparison with other treatments (Table S4). The GI of M. lutariairoparius seed of T1 was 1.35 lower than that of T4 (24.41 vs. 25.76); however, no significant difference was recorded between both treatments (Tables S3 and S4; Figure 3). Furthermore, the subordination value of T1 was only 0.12 lower than that of T4 (0.88 vs. 1.00; Figure 3). In terms of M. sinensis, GR of seed and RSA of seedling were 39.67% and 1.31 cm$^2$, respectively, for the T2 treatment and their subordination values were higher than that of other treatments (Tables S3 and S4; Figure 3). Moreover, the subordination values of GI, RL, and RD of M. sinensis for T2 were 0.93, 0.80, and 0.83, respectively (Figure 3). RD and RSA of hybrid were significantly ($p < 0.05$) enhanced by T3 treatment, with 0.38 mm and 1.12 cm$^2$, respectively (Table S4). In addition, the subordination values of RL, GR, and GI of hybrid were 0.72, 0.92, and 0.99, respectively, for T3. The subordination value of RL for T3 was 0.38 lower than that of T5, whereas the RL of T3-treated hybrid seedling was only 0.90 cm shorter than that of T5-treated hybrid seedling (11.81 cm vs. 12.71 cm; Figure 3; Table S4). Thus, T1 (peat:perlite = 1:1), T2 (peat:perlite = 3:2), and T3 (peat:perlite = 3:1) were the most suitable for M. lutariairoparius, M. sinensis, and the hybrids, respectively.

![Figure 3](image-url)
3.2 | Optimal plug size for Miscanthus

The HI of the five plug sizes for the three Miscanthus genotypes is shown in Figure 4. Compared with the HI of the control, the results showed that the plug size, with the exception of the 128 plugs, had a positive effect on the growth of the Miscanthus seedlings. The HI was composed of plant height, stem diameter, aboveground dry weight, root dry weight, and total plant dry weight. The tendency of these indexes was consistent with the HI among three genotypes (Table S5). The plugs with large volume have improved these indexes (seedling height, stem diameter, aboveground dry weight, and root dry weight) whereas exceedingly small volume plugs have significantly restricted the growth of the Miscanthus seedlings. With the decrease in plug volume, the HI for M. sinensis was reduced. The HI for 32 and 50 plug size was significantly ($p < 0.05$) higher than that 72 and 128 plug size. However, the HI of the 32 plug size was similar to that of the 50 plugs. The aboveground dry weight and total dry weight of 32 and 50 plug size-treated M. sinensis seedlings were significantly ($p < 0.05$) higher than that of 72 and 128 plug-treated ones. No significant difference for HI among the 32 plug size, 50 plug size, and 72 plug size was observed for M. lutarioriparius. For 32 plug size, 50 plug size, and 72 plug size, plant height, stem diameter, root dry weight, and total plant dry weight were not significantly different. The aboveground dry weight of 32 plug size-treated seedlings was significantly ($p < 0.05$) higher than that of 50 and 72 plug-treated seedlings, with only 0.10 and 0.07 higher. In terms of hybrid, The HI firstly increased and then decreased with the reduction of plug volume. The HI of the 72 plug size was the highest and was 0.01 higher than that of the 50 plug size (0.23 vs. 0.24). These indexes of seedling, which include stem diameter, root dry weight, and total dry weight showed similar tendency under plug treatments. In summary, 50 plug size was the optimal amount for M. sinensis. The data for M. lutarioriparius and the hybrid were similar, and 72 plugs were the most suitable.

3.3 | Comparing the field performance of the plants derived from “two-step” seed-derived plugs and rhizomes

Although the survival, overwintering, and establishment rates of the plants derived from “two-step” seed-derived plugs (PS) and rhizomes (PR) on saline-alkali land did not differ significantly, the survival rate of the PS was higher than that of the PR (87.50% vs. 85.94%). However, the overwintering survival rate of the PS was 1.82% lower than that of the RP (91.50% vs. 89.68%). The establishment rates of the PS and PR were similar (78.64% vs. 78.47%; Figure 5). The survival,
overwintering, and establishment rates of seedlings derived from seed directly planted were zero in the field, not being shown in Figure 5.

The trend of the traits measured (biomass yield, plant height, tiller biomass, stem diameter, tiller number, and stem/leaf ratio) for the PS and PR was significantly ($p = 0.03$) influenced by year, and generally increased from 2014 to 2016 (Table 2). At the end of the first and second growing seasons, no significant difference was observed between the biomass yield and yield components (plant height, stem diameter, tiller biomass, and stem/leaf) of the PS and PR. However, during the third growing season, the biomass yield of the PS was 1.2 t/ha, which was significantly ($p < 0.05$) higher than that of the PR (5.0 t/ha vs. 3.8 t/ha; Figure 6A). No significant difference between the stem/leaf of PS and RP was observed, whereas plant height, tiller biomass, and stem diameter of PS were significantly ($p < 0.05$) higher than those of RP (240.00 cm vs. 200.00 cm; 65.00 g vs. 45.00 g; 13.00 mm vs. 11.00 mm; Figure 6B, C, E, and F), respectively. In addition, the tiller number for both PS and RP was similar at the third growing season (Figure 6D).

4 | DISCUSSION

4.1 | The requirement of substrate for Miscanthus seed germination and seedling growth

The substrate is a vital factor in the production of crop seedlings in containers, and its components and properties are important for producing high-quality seedlings. Peat and mushroom substrate can provide abundant nutrients for seedlings. Perlite can maintain substrate moisture. Therefore, the three substrates

| Traits                          | Propagation method | Year | Propagation method × year |
|---------------------------------|--------------------|------|---------------------------|
| Biomass yield (t/ha)            | 0.03               | 0.00 | 0.00                      |
| Plant height (cm)               | 0.51               | 0.00 | 0.00                      |
| Stem diameter (mm)              | 0.58               | 0.00 | 0.00                      |
| Tiller number (n/plant)         | 0.01               | 0.00 | 0.06                      |
| Tiller biomass (g/tiller)       | 0.06               | 0.00 | 0.00                      |
| Stem/leaf                       | 0.60               | 0.00 | 0.71                      |

*FIGURE 6 Performance of the traits of plants derived from “two-step” seed-derived plugs (PS) and rhizomes (PR) in 2014, 2015, and 2016 on saline-alkaline land. (a) Biomass yield, (b) plant height, (c) stem diameter, (d) tiller number, (e) tiller biomass, (f) stem/leaf of PS and PR. Different letters suggest a significant difference at $p = 0.05$. Error bars indicate standard error*
have been compounded which are usually used for nursery of crops, vegetables, and fruits (Santos et al., 2013). Furthermore, a previous study had reported that M. × giganteus stem cuttings in peat/perlite had significantly higher root dry weights than those in other substrates (Hong & Meyer, 2007). However, little is known about the effect of substrates on the seed germination and seedling growth of Miscanthus. The GR, GI, and seedling growth were significantly affected by the substrate treatment, which was consistent with the previous research among other crops (Eudoxie & Alexander, 2011; Nogueira et al., 2012; Oleskog et al., 2000). Our results demonstrated that peat and perlite facilitated the germination of Miscanthus seed. The reason may be that perlite improved the aeration of peat. The nutritional requirements of the seedlings grown from seed were also similar to those of the stem cuttings. The nutritional requirement of the Miscanthus species differs at the seedling stage, but the ratio of the substrate showed a discrepancy, which can be attributed to genotype affect. The structure and mechanical action of the substrate were partly responsible for significant differences in seedling growth under the different treatments. The bulk density of all the treatments was <0.4 g/cm³, according to Abad et al., (2001), who defined an ideal substrate to be with <0.4 g/cm³ which is a good indicator of porosity and thus enabling the free exchange of gas through the growth substrates. In this study, the bulk density of T1 (peat:perlite = 1:1), T2 (peat:perlite = 3:2), and T3 (peat:perlite = 3:1) was lower than that of the other treatments. Moreover, the air-filled porosity and air to water ratio of T1 (peat:perlite = 1:1), T2 (peat:perlite = 3:2), and T3 (peat:perlite = 3:1) was higher than that of the other treatments. Increase in bulk density which subsequently enhances water sorption of the substrate (Barnes et al., 2013). Meanwhile, high air to water ratio improves the aeration of substrate, which subsequently enhances the root growth (Gil et al., 2012). The perlite has improved aeration and water retention capability in case of peat and/or perlite substrate which subsequently contributed toward to the robust seedling growth whereas the spent mushroom substrate was not an optimal choice for the nursery of Miscanthus seed. In terms of the spent mushroom substrate, the primary reason may be that in comparison with perlite and peat, its ability to retain water is poor.

In addition, differences in Miscanthus seed germination and seedling growth can be attributed to the genotype effect. Previous studies have shown that requirements of optimal growth conditions such as germination temperature, light duration, and water content of soil vary from genotype to genotype (Zheng et al., 2017). In this study, the effect of genotype on seed germination and seedling growth was significant. The genetic diversity among Miscanthus species is rich, which has contributed toward difference in substrate requirement for seed germination and seedling growth (Mandic et al., 2014). In case of M. lutarioriparius, the germination conditions were more suitable (Zheng et al., 2017), which subsequently lead to comparatively high seed germination. A previous study indicates that the seedling growth of M. sacchariflorus was excellent in substrates of 1:1 perlite:peat (v/v; Sungchun et al., 2005). Therefore, before choosing the germination conditions, genotype differences should be taken into consideration.

4.2 | Optimal plug size for healthy Miscanthus seedlings

Selection of the suitable plug size not only improves the adaptability of seedlings but also reduces the cost for the large-scale seedlings production. Previous studies showed that the undersized plug size restraints the development and growth of the root. Furthermore, small-sized plugs will carry less quantity of substrate, which leads to poor seedling growth and subsequently affect their adaptability and field performance (Aghai et al., 2014). Moreover, in case of small volume plugs, substrate easily breaks away from roots during transplantation, which has negative impact on seedlings growth and this could be partial reason for higher HI with bigger plug volume. However, an oversized plug size leads to waste of the substrate, with an increase in the production and transportation cost. The plug cell volume of the 32 plug size (140 cm³/plug) is more than twice and three times greater than that of 50 plug size (50 cm³/plug) and 72 plug size (17 cm³/plug), respectively. However, the HI of the 32 plug size, 50 plug size, and 72 plug size was not significantly different for the hybrid. Therefore, we selected the relativity small size to satisfy the production requirements, so that we could reduce the cost and area occupied by the seedlings.

4.3 | The merits of “two-steps” seed propagation for Miscanthus

Previous studies have shown that seed propagation is a promising way to produce large-scale Miscanthus plantations (Clifton-Brown et al., 2016; Hastings et al., 2017; Xue et al., 2015). The “two-steps” seed propagation has substantial potential for the development and expansion of Miscanthus on marginal lands. Currently, there are number of research evidences which suggest that the direct seeds plantation is an unreliable method (Clifton-Brown et al., 2016; Xue et al., 2015). A previous study has reported that the seedlings of M. giganteus grown from seeds had exhibited little competition, which subsequently suffered up to 99.9% mortality within 6 months (Smith & Barney, 2016). In the field, the competitive ability of the seedlings was poor in comparison to the weeds. In this study, a similar result was observed, indicating that the seed and seedling via seeds directly sown lacked adaptability to saline–alkaline soil conditions.

The effective and efficient seed propagation method can promote use of available saline–alkaline soil resource across China for Miscanthus cultivation. The “two-steps”
seed propagation is used instead of directly planting seeds, which has significantly improved the establishment rate of the seedlings. Clifton-Brown et al. (2008) showed that *M. × giganteus* propagated rhizomes have achieved the establishment rate as high as 90% by the end of the first growing season in Sweden. In a field experiment of Boersoma and Heaton (2014), the establishment rate was 66% 2 months after planting. In this study, a 78.64% establishment rate for the seedlings was observed. In addition, no significant difference between the establishment rate of the seedlings via seed and conventional rhizomes propagation was observed. Moreover, overwintering of the seedlings via seed was successful. Although, in the first year in saline-alkali soil, the performance of the *M. lutarioriparius* RP was superior to those of the PS. However, during the next 2 years, the biomass yield and yield components of the PS did not differ significantly from those of the RP. The method of growth of the seedlings during the second year was different than those planted during the first year, and the seedlings emerged from sprouts. It is interesting that in the third growing season, the performance of the PS was better than that of the PR. It could be because during the first growing season, the PS had not developed rhizomes whereas in case of PR, the developed rhizomes had provided nutritional elements for the growth of the aboveground parts (Pyter et al., 2010). During the second and third growing season, peat and perlite surrounding the rhizosphere of the PS may have facilitated the growth of the plants. We hypothesized that peat is rich in organic matter, humic acid, and multiple nutrients, and perlite with microvoids holds the soil water, which offers sufficient nutrients and water for the plants (Haojie et al., 2017). Previous research has indicated that peat can significantly decrease the pH and the saline content of the saline–alkaline soil (Zheng et al., 2008). Therefore, these results indicate that the substrate has facilitated the plant growth through improving the soil, which subsequently lead to better performance of the PS than the PR. However, this finding merits additional research.

4.4 | Challenges associated with the “two-steps” seed propagation for *Miscanthus*

The new method involves some additional logistical steps in comparison with conventional direct seed sowing to plugs, which can lead to high input costs and labor requirements. However, we have recorded number of “blind trays” in case of seeds directly sown to the plugs. Furthermore, a previous study has reported that plugs without seedlings could be up to 40% (Clifton-Brown et al., 2016). This problem hinders mechanized operations in the field. Number of factors such as seed immaturity at harvest, damage to the seed coat during threshing, seed storage conditions, and duration contribute toward the problem. Moreover, the sowing depth has significantly impacted the seed emergence rate of *Miscanthus*, which makes it difficult to use hand or mechanized operations. We used the new method to address these problems. However, future research still needs to be conducted to improve the seed emergence rate of the directly sown trays, lowering the production cost. In addition, peat and perlite are nonrenewable resources; therefore, cost-effective sourcing of the substrate in sufficient quantities could be challenging, especially in case of large-scale plantation. Therefore, renewable products that replace peat and perlite should be identified and studied.

5 | CONCLUSIONS

Commercial rhizome propagation hinders the large-scale cultivation of *Miscanthus*. Seed propagation is key to exploit marginal lands and upscale the *Miscanthus* cultivation. The “two-steps” seed propagation was developed and the evaluation of seedlings adaptability in the saline–alkaline soil was tested at field scale. This provides an important lesson that the genotypes have special technical requirements to plant *Miscanthus* at large scale. In comparison to seeds directly planted and rhizomes directly planted, the seedlings developed with the new propagation method are more adaptable to saline–alkaline soil. In the near future, it could become a feasible *Miscanthus* propagation method that can reproduce via seeds and subsequently facilitates the development and expansion of *Miscanthus* cultivation especially on marginal lands. The ultimate goal of this research work is to reduce the production cost through selecting or treating seeds in a way that will allow direct seed sowing into plugs or field. For future studies, seed cleaning, seed coating, and seed pelleting technologies should be explored to promote efficient and cost-effective seed-based *Miscanthus* propagation.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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