Effects of sand burial and overstory tree age on seedling establishment in coastal Pinus thunbergii forests in the northern Shandong Peninsula, China

by Peili Mao¹,³, Hongxiang Mu², Banghua Cao¹,⁷, Yuhong Liu⁵,³, Zhaofei Fan⁴ and Shumei Wang¹

ABSTRACT
Coastal Pinus thunbergii (Japanese black pine) forests in the northern Shandong Peninsula of China recently experienced widespread natural regeneration failure. This study identifies critical factors that affect natural regeneration of P. thunbergii. Seeds from trees of various ages (13–32 years) were used to investigate the effects of age and burial depth in sand on germination and seedling establishment. Results show that seed density in 2–5 cm soil decreased with increased distance from the shoreline. Sand burial decreased seed germination but did not affect the relative growth rate of seedlings at depths from 0.5 to 3 cm. Germination, leaf mass ratio, and relative growth rates were higher with seedlings originating from older trees, all of which enhanced seedling resistance to sand burial. Tree age and seed burial were found to be determining factors for natural regeneration of the coastal P. thunbergii forest. Silvicultural treatments that promote quality of seed sources and mitigation of sand burial can be used in the future to improve the regeneration of these coastal forests.

Keywords: coastal forest, seed germination, biomass allocation, relative growth rate, seedling establishment

Introduction
Sand burial is the most important selective force driving the distribution and composition of vegetation in coastal sand dune communities and can remarkably influence all aspects of the plant and soil micro-environment, such as vegetation structure, soil temperature, soil moisture, and oxygen levels (Chen and Maun 1999). Burial depth has been demonstrated to have an effect on seed germination (Zhang and Maun 1999). Seeds buried at appropriate depths may have higher germination rates than those on the surface (Chen and Maun 1999, Guo et al. 2010, Liu et al. 2011, Zhu et al. 2014). For example, the highest seedling emergence for wildrye occurred at a 1-cm depth (Zhu et al. 2014), whereas it was 2-cm for Pitcher's thistle (Chen and Maun 1999). Seeds buried at appropriate depths may have higher germination rates than those on the surface (Chen and Maun 1999, Guo et al. 2010, Liu et al. 2011, Zhu et al. 2014). However, excessive burial may prevent germination and subsequent seedling survival. Several authors report that seeds that do not germinate in sand become dormant, especially at deeper depths and form a seed bank, among which a large proportion of seeds die...
The study was carried out at a coastal *P. thunbergii* forest in northern Shandong Peninsula, China (37° 27′ 15″ N, 121° 41′ 57″ E) (Fig. 1). The site has a warm temperate continental monsoon climate with an annual rainfall of 760 mm and mean annual temperature of 11.5 °C. The soil is classed as sandy with low organic matter content (less than 1%). The stand was planted in the early 1970s and a density of 3 000 trees per ha and a canopy coverage of 0.7 were maintained. Average height and diameter at breast height (DBH) was 4.9 m and 7.0 cm, respectively. The understory vegetation is simple and consists of several grass and sedges species, including *Carex rigescens* (Franch.) V. Krecz., *Corispermum stenolepis* Kitag., *Imperata cylindrical* (L.) Beauv. var. Major, saltbush (*Atriplex sibirica* L.), and black nightshade (*Solanum nigrum* L.).

**Materials and Methods**

**Study area**

The soil seed bank was examined in August 2007 using three transects at 200 m intervals set perpendicular to the shoreline.
Fourteen 100 m² plots (10 m × 10 m) were established at intervals of 50 m along each transect. In each plot, five soil samples were collected randomly using a 7-cm diameter cutting ring and separated into two layers (0-2 cm and 2-5 cm with fresh weights of 102 ± 2.0 g and 153 ± 2.4 g, respectively) in which most seeds were buried. The soil within each layer was mixed, stones and litter removed, and soil samples placed in sealed bags for seed determination.

Table 1. Characteristics of *P. thunbergii* mother trees for seed collection

<table>
<thead>
<tr>
<th>Age classes</th>
<th>Diameter at breast height (cm)</th>
<th>Height (m)</th>
<th>Tree age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>9.55–12.74</td>
<td>6.1–7.3</td>
<td>13–17</td>
</tr>
<tr>
<td>Intermediate</td>
<td>12.89–15.92</td>
<td>6.5–9.2</td>
<td>18–23</td>
</tr>
<tr>
<td>Mature</td>
<td>16.56–19.11</td>
<td>6.8–9.8</td>
<td>24–32</td>
</tr>
</tbody>
</table>

Cone and seed collection

Cones were collected from three age classes (13–17 years, 18–23 years and 24–32 years) in stands with densities of 1600–2700 trees per ha in late September of 2008 (Table 1). Tree age was computed based on the age-diameter relationship in equation 1, which was suitable for these source trees (Xu et al. 2003).

(1) \[ A = 10^D (1 - 0.313D^{0.3789}) \]

Where \( D \) is diameter at breast height (cm) and \( A \) is age (years). A sample of 10–15 individuals in each age class was selected. Five to ten cones were randomly collected from each tree and air-dried for four months until open. Seeds were collected and stored for use in the germination experiment.

Seed germination

Germination was carried out at the nursery of the Forestry Bureau of Laishan District in Yantai, Shandong Province. A completely randomized factorial design was used to test the effect of tree age and burial depth on germination and biomass growth. Seeds collected from trees in each of the three age classes: young (13–17 years), intermediate (18–23 years) and mature (24–32 years) were sowed at depths of 0.5, 1.0, 3.0 and 5.0 cm in plastic pots filled with sand taken from where cones were collected (Table 1). The sand was sieved to remove debris and any seeds before use. To calculate the sample mean and variance of the germination rate, 20 seeds were sown in each pot and each treatment replicated five times. During the germination period (4 December 2009–28 April 2010), the minimum and maximum temperatures in the tunnel where the plastic pots were placed varied from -6 °C to 34 °C. All pots were well-watered, and after the first seed germination, germination was assessed every day. The germination rate (Gr%) and mean germination time (MGT in days) were calculated. Gr was calculated as the ratio between the number of germinated seeds at a given time and the number of seeds sown. MGT was calculated as follows (Raccuu et al. 2004):

(2) \[ MGT = \frac{\sum(Dn)}{\sum n} \]

Where \( D \) is the number of days from the beginning of the test, and \( n \) is the number of seeds which germinated on day \( D \). MGT at the 5-cm depth was not calculated due to small number of seeds germinating.

At the end of the experiment (28 April 2010), five randomly selected seedlings were removed from each pot and washed free of sand. Each seedling was divided into leaf, stem, and root components and dried at 75 °C for 48 h. Dry biomass of leaf, stem and root was weighed using an electronic balance (0.1 mg accuracy). Individual seedling biomass (leaf + stem + root mass, g), leaf mass ratio (LMR, leaf mass/seedling biomass, g g⁻¹), stem mass ratio (SMR, stem mass/seedling biomass, g g⁻¹), root mass ratio (RMR, root mass/seedling biomass, g g⁻¹) and root/shoot ratio (RSR, root mass/(leaf + stem biomass)) were calculated.

Seedling growth

Three seedlings with similar heights were retained in each pot; heights and diameters at the surface were measured on 1 May 2010. Heights and diameters were remeasured at the end of the experiment on 30 December 2010. During the experiment, no mortality occurred. The relative height growth rate (RGR₁) and relative diameter growth rate (RGRD) were calculated with equations (3) and (4), respectively.

(3) \[ RGR₁ = \frac{(ln H₂ - ln H₁)}{(T₂ - T₁)} \]

(4) \[ RGRD = \frac{(ln D₂ - ln D₁)}{(T₂ - T₁)} \]

RGR₁ is relative height growth rate (cm cm⁻¹ d⁻¹); RGRD, relative diameter growth rate (mm mm⁻¹ d⁻¹); \( H₁ \) and \( H₂ \) are seedling heights (cm) at initial and harvest periods; respectively; \( D₁ \) and \( D₂ \) are seedling diameters (cm) at initial and harvest periods, respectively; \( T₂ - T₁ \) is the duration of the experiment (days).

Data analysis

Simple linear regression was used to analyze the change of seed density in the soil of 0–2 cm (top layer) and 0–5 cm (top and bottom layers (2–5 cm) combined), while quantile regression was applied to analyze the change of seed density in the soil of 2–5 cm (bottom layer) and the ratio of soil seed density in the top and bottom layers with the distance from the shoreline. Instead of predicting conditional means as simple linear regression does, quantile regression predicts a set of predetermined conditional quantiles or percentiles (95%, 75% and 50%) with the heteroscedastic seed density data (Fig. 2). Effects of burial depth and tree age on seed germination rate (Gr), mean germination time (MGT), seedling biomass, and allometric growth (LMR, SMR, RMR, RGR₁ and RGRD) were first tested using two-way ANOVA as specified in model (5):

(5) \[ Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + \varepsilon_{ijk} \]

Where \( Y_{ijk} \) represents the response variable (Gr, MGT, LMR, SMR, RMR, RGRH and RGRD) from the \( i^{th} \) (i=1,2,3) age class; \( j^{th} \) (j=1,2,3) burial depth and \( k^{th} \) (k=1,2,3,4,5) replication, \( \mu \) is the grand mean, \( A_i \) is the tree age effect, \( B_j \) is the bur-
ial depth effect, \((AB)\) is the interaction of tree age and burial depth and \(\varepsilon_{ijk}\) is the random error. With significant ANOVA tests, post-hoc least-significant difference (LSD) multiple comparisons were further conducted to compare treatment means. Simple linear regression and quantile regression was conducted using the R packages `quantreg` and `stats` and other statistical analyses were conducted in the SPSS for Windows 13.0 (SPSS, Chicago, IL, USA) with the statistical significance level set at \(p = 0.05\).

**Results**

**Seed distribution patterns in soil banks**

Soil seed density of *P. thunbergii* in the 0–2 cm layer and in the entire sampling depth (0–5 cm) increased linearly with the distance from the shoreline, but this positive association was not statistically significant (\(p > 0.1\) and \(R^2 < 0.1\)) due to a large variation in seed density (Fig. 2). As shown by the quantile regression, seed density in the 2–5 cm layer decreased drastically with the distance from the shoreline, although large variations existed within the distance to the shoreline < 200 m. A similar pattern was found with the ratio of seed density between the 2–5 cm and 0–2 cm layers, indicating burial in sand may have more severe effect on soil seed banks at sites near the shoreline.

**Seed germination**

Both burial depth and tree age showed significant differences in \(G_i\) and MGT (Fig. 3, Table 2). \(G_i\) showed a decreased trend,
Biomass decreased with increased burial depth (Fig. 4a). There was also a significant effect of both burial depth and tree age (Fig. 4, Table 2). Biomass of the mature age class decreased more slowly than the other age classes with the increase of depth (Fig. 4a). As depth increased, LMR decreased and RMR and root/shoot ratio increased, while SMR did not change. Among tree age classes, the order of seedling biomass was intermediate < young < mature (P < 0.01). Both the LMR and RMR in the young age class were similar to those in the intermediate age class. However, the LMR in the young and intermediate age classes were lower than that in the mature age class (P<0.01) and the RMR and root/shoot ratio in the young and intermediate age classes was higher than that in the mature age class (P < 0.05). The SMR in the young age class was significant higher than those in the intermediate and mature age classes (P < 0.01; Table 2).

Seedling growth

Significant effects of sand depth on RGRH (F = 1.93, P = 0.16) and GRDR were not found (F = 0.83, P = 0.45; Fig. 5). But RGRH (F = 8.64, P < 0.01) and GRDR (F = 6.31, P < 0.01) were impacted significantly by age classes. The RGRH and GRDR in the youngest class were similar to the intermediate age class, and both were lower than those in the mature age class (P < 0.05). However, there were no significant interactions between depth and age in RGRH (F = 0.89, P = 0.48) and GRDR (F = 0.39, P = 0.81).

Discussion

Determining how to regenerate coastal P. thumbergii forests without altering current forest structure and composition and interrupting its protective function is crucial in coastal forest management (Zhu et al. 2003, 2012). Dense canopies, low soil moisture and thick litter and duff on the ground have long been believed to be the primary factors that hamper the regeneration of P. thumbergii (Zhu et al. 2012). However, recent studies found that few seedlings were found in stands near the shoreline, despite having greater light intensity (Zhang et al. 2009). Besides high soil salinity, one potential factor overlooked before could be low seed density and sand burial as observed in our study. There were fewer seeds in the soil near the shoreline (< 200 m), and moreover, more seeds were distributed in the 2–5 cm layer due to sand burial (Fig. 2). As demonstrated by the germination experiment, fewer seeds of P. thumbergii could germinate at a depth > 3 cm (Fig. 3a). Therefore, low seed density and depth may contribute to the poor regeneration of P. thumbergii forest near the shoreline.

Table 2. Two-way ANOVA analyses of effects of sand burial, tree age and their interaction on seed germination and seedling biomass allocation of P. thumbergii.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sand burial depth</th>
<th>Tree age</th>
<th>Sand burial depth × Tree age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr</td>
<td>210.69 &lt;0.01</td>
<td>7.40 &lt;0.01</td>
<td>2.12 0.07</td>
</tr>
<tr>
<td>MGT</td>
<td>212.34 &lt;0.01</td>
<td>58.98 &lt;0.01</td>
<td>13.19 0.01</td>
</tr>
<tr>
<td>Biomass</td>
<td>32.32 &lt;0.01</td>
<td>24.22 &lt;0.01</td>
<td>3.27 0.05</td>
</tr>
<tr>
<td>LMR</td>
<td>9.82 &lt;0.01</td>
<td>14.07 &lt;0.01</td>
<td>1.32 0.28</td>
</tr>
<tr>
<td>SMR</td>
<td>1.27 0.29</td>
<td>10.76 &lt;0.01</td>
<td>0.63 0.64</td>
</tr>
<tr>
<td>RMR</td>
<td>13.22 &lt;0.01</td>
<td>6.90 &lt;0.01</td>
<td>0.47 0.76</td>
</tr>
<tr>
<td>Root/shoot ratio</td>
<td>13.62 &lt;0.01</td>
<td>6.72 &lt;0.01</td>
<td>0.57 0.69</td>
</tr>
</tbody>
</table>

Fig. 3 Effects of sand burial and mother tree age on seed germination (Gr) and mean germination time (MGT) of P. thumbergii. Values are means ± SD.
shoreline. The concomitant relationship between soil seed bank and plant regeneration was also previously reported (Zhu and Wang 1992).

Burial depth influences seed germination and seedling establishment (Chen and Maun 1999, Redondo-Gómez et al. 2011, Zhu et al. 2014). Plant species respond differently to burial depths in seed germination (Chen and Maun 1999, Zheng et al. 2005, Li et al. 2007). In this study, *P. thunbergii* seeds showed the greatest germination rates at depths of 0.5 and 1 cm (Fig. 3a). Liu et al. (2011) and Zhu et al. (2014) also found similar results and thought they were related to greater humidity (soil moisture) around these seeds in the top layer. The lowest seed germination rates at the 5 cm burial depth may be related to low oxygen in the sand (Vleeshouwers 1997) or the exhaustion of seed reserves during germination (Chen and Maun 1999, Liu et al. 2011). Thus, it could be

![Fig. 4. Effects of sand burial and mother tree age on seeding biomass allocation of *P. thunbergii*. Values are means ± SD.](image)

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Seed quality might be increased by ovule abortion monotonically with tree age (Chen and Maun 1999) and chances of infection by seed borne and soil borne pathogens (Gómez et al. 2011). The delayed germination will increase the chances of infection by seed borne and soil borne pathogens (Beckstead et al. 2010), related to low germination rates at approximately 5 cm depth.

Tree age significantly affected seed germination and has been reported in other studies. Germination rates for Sorbus torminalis (L.) (Espahbodi et al. 2007) and P. echinata Mill. Acc. (Grayson et al. 2002) increased at first and then declined with tree age. The maximum rate appeared in the intermediate age class (30 cm class for S. torminalis and 33 cm class for P. echinata, respectively). Similarly, Lyngdoh et al. (2014) found that seeds collected from intermediate aged trees (i.e., 45 years old) of Livistona jinkensiana Griff. consistently had greater weight and germination rates compared to seeds collected from trees 18–35 years of age, and 54–74 years of age. However, the germination rate increased and MGT decreased monotonically with tree age of P. thungergii in this study (Fig. 3). Seed quality might be increased by ovule abortion rate of cones with increasing tree age (Mao et al. 2010). Besides high rates of embryo abortion in mature seeds of older Cistus albidus L., increasing salicylic acid, jasmonic acid and vitamin E levels also were important to ensure seed quality (Müller et al. 2014). Furthermore, we found significant interaction on MGT between depth and tree age. Age slowed down the rate of increase of MGT with the increase in depth. Cendán et al. (2013) thought optimizing the timing of germination was vital for seedling establishment in the natural environment. These results serve as an example of the complexity of seed germination in natural environments.

Biomass allocation patterns of plant species in different environmental conditions demonstrate their adaptive strategies (Poorter et al. 2012, Zheng et al.). High root biomass investment is an important adaptation for plants to survive under sand dune conditions (Gad et al. 2012). In this paper, we found RMR and root/shoot ratio increased with increased depth. When a plant is buried, soil moisture in the root zone increases and soil temperature decreases (Shi et al. 2004). Moreover, high root biomass allocation benefits water absorption (Perumal et al. 2006). However, some researchers reported that sand burial mainly increased aboveground biomass of seedlings (Gilbert and Ripley 2010, Xu et al. 2013a). High investment in stem biomass could increase stem elongation of seedlings, making them less vulnerable to burial (Brown 1997, Xu et al. 2013a). Frosini et al. (2012) did not find the root/shoot ratio of Sporobolus virginicus (L.) Kunth was affected significantly by sand burial. Sykes and Wilson (1990) reported six of 29 New Zealand sand dune species decreased root/shoot ratios with depth, four species did not change significantly, while 19 species increased root/shoot ratios. These results indicated biomass allocation responses to sand burial vary by species and their growing conditions. In this study, LMR increased with tree age (Fig. 4b). High investment in leaf biomass was advantageous for species to maintain high photosynthetic capacity and fast growth (Harris and Davy 1987, 1988; Zheng et al. 2012) and improve light interception ability in the understory (Poorter et al. 2012). The increase of leaf biomass investment with age is an important adaptive strategy for P. thungergii seedlings to respond to sand burial and low light.

Growth is used as a predictor because it integrates the effects of different stresses on species vigor and carbon balance (Pedersen 1998). Although seedling biomass decreased with an increase in depth at the beginning of the experiment, no significant differences were found at 0.5 to 3.0 cm depths at the end of the experiment (Fig. 5), indicating that partial burial might stimulate seedling growth. Many plant species enhanced seedling growth by partial burial compared to complete burial (Shi et al. 2004, Zheng et al. 2012). For

Fig. 5. Effects of sand burial and mother tree age on seeding relative growth rate of P. thungergii. Values are means ± SD.
instance, high soil moisture and low temperature around root zones by partial burial benefited water absorption and high leaf water content, which elevated the photosynthesis and growth of *Ulmus pumila* L. seedlings (Shi et al. 2004). Perumal and Maun (2006) postulated that the growth stimulation of partially buried seedlings was caused primarily by an increase in root growth. Plant species survival to sand burial could maintain a trade-off between height growth and mechanical stability (Niklas et al. 2006, Vospernik et al. 2010). Increased stem elongation could make plants more prone to wind-induced drag (Anten et al. 2005). However, this risk could be mitigated by increasing stem thickness at the same time (Goodman and Ennos 1996, Xu et al. 2013a), which increases the ability of plants to resist mechanical stress (Xu et al. 2013b). Both $R_{GRH}$ and $R_{GRD}$ in the mature age class were greater than those in the young and intermediate classes (Fig. 5), indicating advantages on both the growth of stem height and stem thickness. With the increase of tree age, the resistant ability to sand burial for *P. thunbergii* seedlings increased. Moreover, high growth rates provide a competitive advantage for species to grow the competition of surrounding species (Toledo-Aceves and Swaine 2008). A higher growth rate is another important adaptive strategy for light-demanding *P. thunbergii* seedlings to respond to sand burial and low light conditions under the forest canopy.

Sand burial was an important stimulating factor on plant regeneration. However, suitable depths (e.g., 0.5–3.0 cm) need to be determined for improved seed germination and seedling growth for species such as *P. thunbergii*. Overstory tree age of *P. thunbergii* exhibits a significant effect on seed regeneration and seedling growth as well. Older trees produced quality seeds with higher germination rates. Moreover, seedlings from these seeds showed high growth rates and light interception ability due to high leaf biomass investment. Large variation in seed germination and seedling growth among different age classes and burial depths suggests that other biotic and abiotic factors may also play an important role in *P. thunbergii* regeneration and growth. The interaction of tree age and burial depth with other limiting factors such as light intensity, soil moisture, and soil nutrients on natural regeneration should be addressed in future studies to better understand the natural regeneration processes of this species. Furthermore, light thinning is needed to improve the vigor and growing conditions of residual trees for the production of high quality seeds while maintaining the protective functions of these forests. Light thinning also has beneficial effects on the growth of ground vegetation and mitigation of excessive sand burial. While further research is necessary, resource managers can use the information and suggestions provided here to enhance regeneration rates of these forests as the function of dune forests is vital for the mitigation of damage to dune/coastal areas.

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