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Sand Priming Promotes Seed Germination, Respiratory Metabolism and Antioxidant Capacity of *Pinus massoniana* Lamb.

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Abstract: The validity to promote seed vigor of sand priming had been reported; however, its feasibility in pine seeds remained unknown. In this study, Masson pine (*Pinus massoniana* Lamb.) seeds of three varieties were used to investigate the effect of sand priming on seed germination, respiratory metabolism and antioxidant capacity. Seeds treated with hydrated sand, about 5% moisture over 2 day, were employed as the priming group with non-primed seeds as the control. The germination test and field test showed that the germination rate, germination potential, vigor index and field emergence rate of sand-primed seeds were significantly enhanced by 8.3~12.3%, 3.9~11.5%, 40.4~72.3% and 5.8~8.9% compared to non-primed seeds. The oxygen-sensing test indicated that sand priming accelerated seed respiration. Besides these, antioxidant enzyme activities and endogenous GA_1 and IAA are also enhanced after sand-priming treatment. Furthermore, the variation tendency of gene expression of *GID1*, *POD* and *SDH* was in line with the physiological parameters. Based on the results, the regulatory mechanism of germination promotion of the Masson pine by sand priming was clarified. Sand priming might promote respiratory metabolism, endogenous hormones and antioxidant capacity through influencing the related gene expression, which would have a conjoint promotion on the seed germination of *Pinus massoniana*.

Keywords: *Pinus massoniana*; sand priming; seed germination; respiratory metabolism; antioxidant capacity

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1. Introduction

Masson pine (*Pinus massoniana* Lamb.) is an economical and eco-admirable evergreen conifer in China. The eighth national forest inventory revealed that the area of the Masson pine was 10.01 million hm^2, accounting for 6.08% of the total arboreal forest area [1]. Owing to the characteristics involving fast growth, high yield, strong adaptability, as well as comprehensive utilization, the Masson pine played crucial roles in forest ecosystem, afforestation and re-vegetation projects [2]. Seeds are the basis of seedling cultivation of the Masson pine; however, seeds become aged and deteriorated, accompanied by damage of the cell membrane function and some enzymatic activities, during storage, which severely restricts the afforestation program of *Pinus massoniana*. In this regard, how to ameliorate seed performance of *Pinus massoniana* becomes a difficult problem in production practice. Seed priming is a high-efficiency and easily operated method for enhancing the seed vigor, seedling uniformity and stress resistance [3,4]. It is a seed technology carried out by moisture absorption then re-drying without radicle emergence. Multiple seed priming techniques have been developed. PEG (polyethylene glycol) priming promotes the germination characteristics of barley seeds under drought conditions [5], while KNO_3 priming enhances canola seed performance when subjecting salt stress [6]. Sodium nitroprusside as chemical priming agents had a well-recorded property for increasing the capacity of stress

tolerance of *Ocimum basilicum* L. [7]. Bio-priming with *Trichoderma harzianum* reduced the damage of salt stress to bread wheat [8].

However, the above methods were not suitable to apply in treating a large quantity of seeds because huge cost or special equipment was required. The use of the above methods is complicated, making it difficult to popularize the technology for ordinary farmers. Solid matrix priming combines the seeds, water and organic/inorganic solid material with a proper ratio at a certain temperature [9,10]. Sand as a solid matrix has the definite capacities to hold water and release water to seeds slowly, and it is cheap, easily separated and can be repeatedly utilized. In practice, sand priming was not only employed for improving the germination rate under stress conditions of *Oryza sativa* L. [11] but also *Medicago sativa* L. [12]. As mentioned above, priming promotion usually occurred in the germination stage and young seedling establishment, which was in line with the research [13].

Over-accumulation of ROS (reactive oxygen species) has a negative effect on proteins, lipids and carbohydrates, as well as DNA, and might further give rise to the oxidative damage of the organisms or plant cells [14]. Proline was accumulated extensively in plants under stress conditions to stabilize the structure of biomacromolecules and improve the stress tolerance [15]. MDA (malondialdehyde) was commonly employed for reflecting the lipid peroxidation levels and the antioxidant status in plant cells [16]. For self-preservation, plant cells have evolved defensive system to minimize the damage of ROS over-accumulation, such as SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), APX (ascorbate peroxidase), GPX (glutathione peroxidase) and so on [17,18]. Sand priming enhanced the activities of CAT, SOD, APX and POD and inhibited MDA gathering during the germination stage [19]. The afforestation program needs both economic efficiency and practical feasibility. Previous research had proved that sand priming was a valid pretreatment to promote seed germination in some field crops. Therefore, a hypothesis was proposed about whether sand priming could be applied to promote seed germination of *Pinus massoniana*. In order to evaluate the effects of sand priming *Pinus massoniana*, seed respiration capacity (by oxygen-sensing technique and SDH activity measurement), main antioxidant enzymes (POD, CAT and SOD) and endogenous phytohormones (GA_1, IAA and ABA) between sand-primed seeds and non-primed seeds were analyzed. To further clarify the mechanism at the gene level, several related genes were determined to verify the possible regulatory mechanism of the germination promotion of *Pinus massoniana* by sand priming. The objective of this study will provide a new and efficient pretreatment of *Pinus massoniana* seeds before germination.

2. Materials and Methods

2.1. Plant Materials

The seeds of Masson pine varieties "Dai 1.0", "Dai 1.5" and "Dai 2.0" were obtained in 2017 from Laoshan forestry center in Hangzhou, China, located at 29°50′35″ N, 119°04′01″ E. Laoshan forestry center preserves the most important germplasm resources of *Pinus massoniana* Lamb. in China. The above three varieties of seed orchard of Masson pine have gradually improved, and they have been widely used in genetic improvement and afforestation. The landform of this region is mainly low mountains and hilly, and it has a subtropical monsoon climate with 1063 mm of average annual precipitation and 17~18 °C of mean temperature. To break dormancy, seeds were put into zippered plastic bags and subjected to moist chilling at 4 °C at 35% moisture content for 60 d in the dark. The initial germination rate ranged from about 10–40%, which was increased to approximately 20–65% after moist chilling. Finally, seeds were stored at 4 °C for 3 months before experiment.

2.2. Sand-Priming Treatment

The objective of this experiment was to address whether and how sand priming promotes seed germination of *Pinus massoniana*. The priming treatment followed [12] with slight modifications. Sand was screened by sieve (d = 2 mm) to remove the large sand particles and sterilized by oven at 130 °C for 2 h. Each gram of seeds was mixed with 100 g

of sand, about 5% moisture and then sealed by preservative film in plastic boxes at 20 °C for 48 h. It was conducted in dark conditions to minimize the influence of light. Post priming, seeds were laid on the dry filter paper to dehydrate until initial moisture level. Except for priming procedure, all conditions between sand-primed seeds and non-primed seeds were absolutely identical. Before germination test, a portion of seeds were instantaneously frozen then stored at −80 °C for oxygen-sensing test, antioxidant capacity and endogenous hormone determination.

2.3. Laboratory Germination Experiment

Three independent replicates with 100 seeds per replicate were conducted in germination test. Seeds were evenly floored on fully hydrated filtration paper at 25 °C/dark. The quantity of germinated seeds was recorded until there was no further germination (12 d). Germination rate (GR), germination index (GI), vigor index (VI) [20] and the mean germination time (MGT) [11] were calculated according to the following formulas:

GR = the number of germinated seeds within the 12 days/the total number of seeds × 100%.

$$GI = \sum (Gt/Dt)$$

here, Gt is corresponding number of seeds germinated in the t day; Dt is time corresponding to Gt in days.

$$VI = GI \times S$$

here, GI refers to germination index. S is the average length of 10 seedlings.

$$MGT = \sum T_i N_i \; / \; \sum N_i$$

here, N_i refers to the number of the new germination seeds in day T_i.

$$\text{Germination potential} = n_1 \text{ within 5 days}/n_2 \times 100\%$$

here, n_1 represented the number of germinated seeds; n_2 represented the number of totally tested seeds. The seedlings of 3, 6, 9 and 12 d were collected and stored at −80 °C for RNA extraction.

2.4. Field Emergence Rate Test

The field emergence rate test was conducted at the Guantang Farm of Zhejiang Agriculture and Forestry University (ZAFU) located in lin'an, Hangzhou, Zhejiang, China for 21 days in May 2018. In the 10 cm soil layer, the average temperature was 16.8 °C, and the average relative water content was 67.9%. The field emergence rate test was conducted using a complete random design on three replicates of 100 seeds per sample. The plant and row spacing was 20 cm × 30 cm. Seeds were sown in 2 cm deep soil covered with 1~2 cm deep plant ash. The number of seedlings was recorded every two days after emergence until 21 d after sowing.

2.5. Oxygen-Sensing Measurement

Oxygen-sensing technology was found that was an effective and rapid method in seed vigor assessment of *Pinus massoniana* [21]. In this study, we wanted to clarify whether sand priming could enhance the seed vigor by strengthening the seed respiration of *Pinus massoniana*. There were three replicates, and each replicate contained 50 seeds in oxygen-sensing test. The seeds were airproofed with photosensitive material and oxygen content was measured at hourly intervals by Q2 instrument (ASTEC Global, Maarssen, The Netherlands). After 12 days of monitoring, four oxygen-sensing parameters (1-IMT: increased metabolism time; 2-OMR: oxygen metabolism rate; 3-COP: critical oxygen pressure; 4-RGT: relative germination time) were calculated by oxygen-sensing software (ASTEC Global, Maarssen, The Netherlands) [21].

2.6. Antioxidant Capacity Assessment

Antioxidant capacity assessment was established in triplicate and on 1 g seeds for each sample. Superoxide dismutase (SOD) activity was measured by determining the inhibition to photochemical reduction of Nitroblue tetrazolium as monitored at 560 nm, catalase (CAT) activity was measured by the absorbance decrease at 240 nm based on the H_2O_2 decomposition at 25 °C and peroxidase (POD) activity was measured by monitoring the increase in absorbance at 470 nm due to guaiacol oxidation at 25 °C [22]. Succinate dehydrogenase (SDH) activity was determined by mg of SDH per g protein an hour using the extinction coefficient 1.03×10^3/M/cm at 420 nm [23]. The content of Malondialdehyde (MDA) was calculated by the formula "μmol MDA g^{-1} FW = 6.45 ($OD_{532} - OD_{600}$) − 0.56 × OD_{450}"; proline content was assayed by the spectrophotometer at 520 nm [24].

2.7. Endogenous Hormones Determination

For endogenous hormone determination, three biological replicates were conducted, and 0.5 g seeds were used for each replicate. The cryopreserved seed embryos of *Pinus massoniana* were ground to powder. Based on the 1:10 ratio (*M*/*V*), 0.5 g of powder was added into 5 mL of 80% methanol extract in the centrifuge tube. During this period, vortex once every half an hour and repeat it 5 times. After standing for 12 h at 4 °C in the dark, centrifugation at 12,000× *g* at 4 °C for 20 min was conducted to separate of precipitate. A volume of 500 μL of 30% methanol solution was used to dissolve the precipitate. Centrifuge at 12,000× *g* for 15 min at 4 °C to collect the precipitate and store it in the sample vial. Endogenous gibberellin A1 (GA_1), abscisic acid (ABA) and indole-3-acetic acid (IAA) were measured by LC-ESI-MS/MS [25]. MS system: ion spray voltage −4500 V, temperature 550 °C, curtain gas 40 psi, gas 1:50/gas 2:50. Chromatographic system: HSS T3 liquid chromatography column (100 × 2.1 mm, 1.8 μm), mobile phase A (0.1% formic acid-aqueous solution), mobile phase B (0.1% formic acid-acetonitrile) and sample volume = 5 μL.

2.8. Quantitative Real-Time PCR Assay

Total RNA was extracted using Spin Column Plant Total RNA Purification Kit (Sangon Biotech, Shanghai, China). cDNA was synthesized from 1000 ng of total RNA according to reverse transcription kit (Takara, Beijing, China). The sequences of 3 candidate genes were acquired from the GenBank. Gene annotation and primer sequence were summarized in Table 1. qRT-PCR was performed in three biology replications and two technique replications by TB Green Premix Ex Taq (Takara, Beijing, China), and the result was evaluated by $2^{-\Delta\Delta Ct}$ method.

Table 1. The primer sequence and annotation information of candidate genes used in this study.

Accession Number	Primer Sequence (5′–3′)	Gene Annotation
MG755268.1	GAACCCTATGTTCGGAGGCG CCGAAGGGATTACAGGCTGG	Gibberellin metabolism protein gene (*GID1*)
KF910089.2	GGTTGTGTTGTCTGGCGAAC GTCTTATTGTCCCCGGGCTT	Peroxidase gene (*POD*)
KM496547.1	ATTCTCTGCAGATCGGCCTC TGCGATCGCGTAGGAAAGAG	Succinate dehydrogenase gene (*SDH*)
KM496540	AATCAACCCTGCATCTCGTC CTGGATCTTGGCCTTGACAT	Polyubiquitin 4 gene (*UBI4*)

2.9. Data Analysis

Two-way analysis of variance was carried out with SPSS 19.0 (IBM SPSS Statistics, Chicago, IL, USA). The effect of treatment and variety are recognized as the first and second factors. Duncan's multiple range test was employed to determine if there were significant differences among different treatments at $p < 0.05$.

3. Results

3.1. Effect of Sand Priming on Seed Germination Traits and Field Emergence of Pinus massoniana Lamb.

The major purpose of this experiment was an attempt to clarify the effect of sand priming on the seed germination and field emergence of *Pinus massoniana*. From Table 2, it can be observed that the vigor of "Dai 2.0" appeared to be very high; the germination rate was over 73.6% without priming, while "Dai 1.5" and "Dai 1.0" were only 48.5% and 22.4%, respectively. The results showed that sand priming at 25 °C for 48 h significantly improved seed germination and field emergence among all of three Masson pine varieties (Table 2). For "Dai 2.0" seeds, the germination rate, germination potential, germination index and vigor index and field emergence rate were 1.14, 1.57, 1.29, 1.40 and 1.14 times the non-primed seeds, respectively. For "Dai 1.5" seeds, the above indicators were 1.17, 1.61, 1.31, 1.47 and 1.14 times. For "Dai 1.0" seeds, the above indicators were 1.55, 1.85, 2.27, 1.72 and 1.46 times. Meanwhile, the mean germination time was shortened about 0.6–1.2 day. Interestingly, by analyzing the data, we found that the most significant promotion occurred on low-vigor seeds (Dai 1.0), and the least significance occurred on high-vigor seeds (Dai 2.0). Therefore, we speculated there was a negative correlation between the degree of promotion of sand priming and the seed vigor itself.

Table 2. Effect of sand priming on seed germination of *Pinus massoniana* Lamb.

Variety Name	Treatment	Germination Rate (%)	Germination Potential (%)	Germination Index	Vigor Index	Mean Germination Time (d)	Field Emergence Rate (%)
Dai 2.0	NP	73.6 ± 3.2 b	20.2 ± 3.8 b	8.21 ± 0.20 b	18.68 ± 0.81 c	4.6 ± 0.2 d	63.1 b
	SP	84.0 ± 4.0 a	31.7 ± 3.7 a	10.56 ± 0.43 a	26.23 ± 0.77 a	4.0 ± 0.3 e	72.0 a
Dai 1.5	NP	48.5 ± 3.5 d	11.6 ± 1.6 c	6.39 ± 0.11 c	16.48 ± 1.18 d	5.8 ± 0.4 c	43.2 c
	SP	56.8 ± 3.2 c	18.7 ± 1.3 b	8.34 ± 0.45 b	24.30 ± 1.30 b	5.0 ± 0.1 d	49.0 c
Dai 1.0	NP	22.4 ± 2.5 f	4.6 ± 0.6 d	2.96 ± 0.39 d	6.13 ± 0.37 f	9.0 ± 0.5 a	18.9 e
	SP	34.7 ± 3.3 e	8.5 ± 1.5 cd	6.73 ± 0.62 c	10.56 ± 0.69 e	7.8 ± 0.3 b	27.7 d

Note: NP and SP represent non-primed seeds and sand-primed seeds, respectively. Means of different treatments followed by the same lowercase letters are not significantly different at 5% probability level, according to Duncan's multiple range test.

3.2. Effect of Sand Priming on Seed Respiration of Pinus massoniana

An oxygen-sensing test was conducted to reveal the dynamic changes of respiration metabolism at the initial stage of seed germination. The research [21] indicated that IMT, COP and RGT negatively reflected the germination rate, whereas OMR was in accord with the laboratory germination performance. In this paper, the OMR value of sand-primed group was significantly increased by 5.0~20.0%, whereas IMT, COP and RGT values were lower than those of non-primed seeds (Table 3). Interestingly, SDH participated in step six of the TCA cycle as well as the respiratory chain, which might reflect respiratory capacity to some extent. SDH activity was promoted by 211.6~265.0% (Figure 1) in subsequent experiments. Therefore, the opinion that sand priming improves seed respiration of *Pinus massoniana* could be verified.

Table 3. Effect of sand priming on seed respiration of *Pinus massoniana*.

Variety Name	Treatment	IMT (h)	OMR (%/h)	COP (%)	RGT (h)
Dai 2.0	NP	32.25 ± 3.15 ab	0.84 ± 0.05 b	30.97 ± 1.75 c	183.11 ± 5.38 b
	SP	25.15 ± 2.89 c	0.99 ± 0.08 a	25.17 ± 2.08 e	146.67 ± 5.77 d
Dai 1.5	NP	33.83 ± 4.47 ab	0.77 ± 0.01 c	33.18 ± 2.54 b	188.24 ± 8.59 b
	SP	29.25 ± 3.95 bc	0.82 ± 0.02 b	28.01 ± 3.12 d	159.47 ± 10.24 c
Dai 1.0	NP	36.28 ± 3.82 a	0.75 ± 0.05 c	34.97 ± 2.24 a	199.73 ± 11.16 a
	SP	31.69 ± 2.93 ab	0.95 ± 0.07 a	27.57 ± 3.36 d	163.22 ± 17.47 c

Note: NP and SP represent non-primed seeds and sand-primed seeds, respectively. IMT: increased metabolism time; OMR: oxygen metabolism rate; COP: critical oxygen pressure; RGT: relative germination time. The same lowercase letters do not differ significantly at 5% probability level.

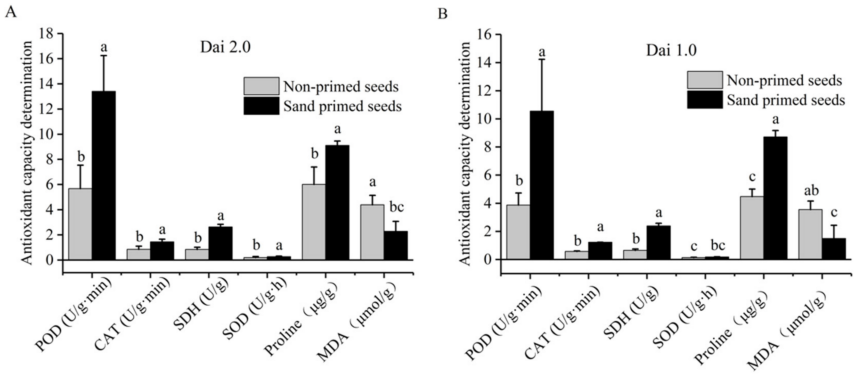

Figure 1. Effects of sand priming on enzymatic activities and the content of proline and MDA of *Pinus massoniana*. (**A**) The result of Masson pine variety "Dai 2.0". (**B**) The result of Masson pine variety "Dai 1.0". The same lowercase letters are not significantly different at $p < 0.05$, according to Duncan's multiple range test. MDA: malondialdehyde; CAT: catalase; SOD: superoxide dismutase; POD: peroxidase, SDH: succinate dehydrogenase.

3.3. Effect of Sand Priming on Antioxidant Capacity of Pinus massoniana

Sand priming at 25 °C for 48 h improved the antioxidant capacity of both high-vigor seeds and low-vigor seeds (Figure 1). For "Dai 2.0", the activities of POD, CAT and SOD after sand priming were increased by 135.9%, 70.8% and 39.4% compared with the non-primed controls (Figure 1A). For "Dai 1.0", the enhancement was more obvious; POD and CAT activities were enhanced by 172.3% and 112.8%, respectively (Figure 1B). For the SOD activity, there was a little discrepancy (39.4% > 32.6%), but it was not significant. Moreover, the proline content of "Dai 2.0" was increased by 51.3%, and MDA content was reduced by 48.0% after sand-priming treatment (Figure 1A). In "Dai 1.0", proline content was increased by 94.6%, whereas MDA content was reduced 57.9% (Figure 1B). In general, the above results showed that sand priming could invigorate the antioxidant capacity of *Pinus massoniana*.

3.4. Effect of Sand Priming on the Content of Endogenous Phytohormone

During seed germination and young seedling establishment, GA and ABA played the role of a switch, controlling the seeds' germination or dormancy, and IAA could also promote seed germination in some content. The content of endogenous phytohormones (IAA, GA_1, ABA) of sand-primed seeds and non-primed seeds was determined. Our results showed that the content of IAA and GA_1 of "'Dai 2.0" after sand priming was significantly enhanced by 24.9% and 74.3%, and ABA content was reduced by 48.6% (Figure 2A). For "Dai 1.0", IAA and GA_1 were notably increased by 30.9% and 116.0%, whereas ABA was significantly reduced by 56.6% (Figure 2B). In light of the antagonism or synergistic action among ABA, GA_1 and IAA, the investigation demonstrated that sand-priming treatment could promote the synthesis of IAA and GA_1 and inhibit the synthesis of ABA.

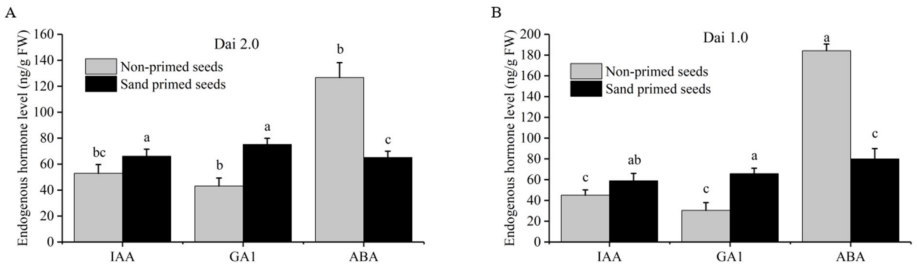

Figure 2. Effects of sand priming on the content of endogenous phytohormone of *Pinus massoniana*. (**A**) The result of Masson pine variety "Dai 2.0". (**B**) The result of Masson pine variety "Dai 1.0". IAA: indole-3-acetic acid; ABA: abscisic acid; GA_1: gibberellin A1. Lower-cased letters indicate statistically significant differences ($p < 0.05$, Duncan multiple range test).

3.5. Effect of Seed Priming on Gene Expression

In view of the significant improvement of sand priming on seed germination, antioxidant capacity as well as endogenous hormones content, the change in molecular level aroused our curiosity. Three relatively regulatory genes (*POD*, *SDH* and *GID1*) related to antioxidant capacity, seed respiration and hormone response were selected for quantification analysis. To investigate the dynamic changes of the effect of sand priming on gene expression, "Dai 1.0" seedlings of 3, 6, 9 and 12 days were collected to perform qRT-PCR analysis. The number represents the multiple of the expression level (sand-priming group/non-priming group), and ">1" means upregulation while "<1" means downregulation (Figure 3). The results indicated that all candidate genes were upregulated after sand priming on the third day of germination, ranging from 1.37 to 2.42 folds. *SDH* peaked on the third day. On the sixth day, the promotion of *GID1* and *POD* reached the peak and were 3.2 and 8.43 folds, respectively. On the 9th and 12th day, the promotion effect was not so obvious. Based on the above results, we speculated that the acceleration of sand priming on genes expression mainly manifested in the early stage of seed germination, and the general trend increased first and then decreased.

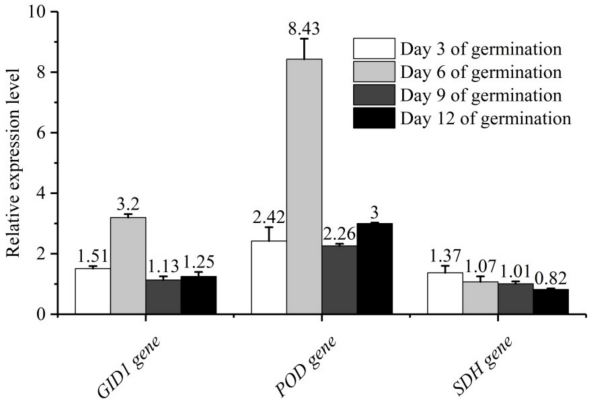

Figure 3. Effect of sand priming on genes expression in *Pinus massoniana* "Dai 1.0". Annotation information of above genes had been listed in Table 1.

4. Discussion

The demand for high-quality seeds has become a necessary priority to face the present requirement for high standards in the afforestation project of *Pinus massoniana* Lamb. Seed priming is an excellent choice and well-suitable technique to improve seed quality, which could promote seed germination and ensure uniform seedling establishment [26]. Moreover, seeds after priming showed higher activities of antioxidant enzymes and lower levels of ROS [27]. Sand priming had been examined in *Oryza sativa* L., *Medicago sativa* L., *Zea mays* L. and *Gossypium hirsutum* Linn. [11,12,28,29], but the reports on promoting seed germination of *Pinus massoniana* were little.

Our study showed that seed germination of *Pinus massoniana* was improved, and the mean germination time was shortened among all of three varieties (Table 2), which was consistent with those in *Oryza sativa* and *Solanum melongena* L. [3,11]. The research [11] reported that the germination rate and germination potential of sand-primed seeds were increased by 4.2~6% and 4.2~8% compared to non-primed controls; meanwhile, the mean germination time was decreased by about 0.01~0.04 d. The research [3] also verified that seed priming could enhance the germination rate and reduce germination time simultaneously. Besides for promoting seed germination and shortening germination time, this study indicated that the stimulation of sand priming was more visible for low-vigor seeds (Dai "1.0") than high-vigor seeds ("Dai 2.0").

ABA and GA act as antagonistic elements to determine the seed fate that results, dormancy or germination [30]. IAA can promote seed germination of *Cunninghamia lanceolata* (Lamb.) Hook. [31]. Additionally, exogenous GA_3 could promote seed germination and

seedling vigor of *Pinus massoniana* by strengthening seed respiration, stimulating IAA and GA_1 biosynthesis and reducing the ABA level [32]. In the current study, sand priming also influenced these endogenous hormones in a positive manner to promote seed germination. A previous study [33] indicated that seed priming alleviated the disadvantages caused by stress conditions, which resulted in a low ABA concentration and augmentation of salicylic acid (SA), IAA and GA. Collectively, a conclusion can be drawn that plant hormones might play vital roles during seed germination of *Pinus massoniana* after sand priming.

The study [34] indicated there was an intricate interplay between phytohormones and ROS during abiotic stress conditions. IAA joined in ROS homeostasis by reducing the ROS to promote plant growth [35], while ABA as a stress trigger controlled stomatal closure by H_2O_2, which was generated in the ROS metabolism [36]. This study indicated that sand priming could increase the antioxidant capacity and stress tolerance of the Masson pine by increasing the activities of antioxidant enzymes (CAT, POD and SOD) and proline content and decreasing MDA content (Figure 1). Similarly, H_2O_2 priming could cause an enhancement of the activities of APX, SOD, CAT and GPOX and decrease the MDA content [37].

Sand priming played key roles in the regulation of seed germination, antioxidant capacity and physiological indexes of *Pinus massoniana*, but the molecular events underlying also needed to be made clear. *GID1* gene, as a key regulatory element in the GA signaling pathway, affects seed germination induced by GA [38]; *POD* gene regulates the antioxidant capacity by encoding peroxidase, which participates in scavenging ROS [39]. In this paper, the endogenous GA and IAA (Figure 2) and the activities of POD, CAT and SOD (Figure 1) were increased, which was broadly in line with the highly expressed *GID1* and *POD* (Figure 3). *SDH* gene participated in the TCA cycle (tricarboxylic acid Cycle) and may function as a regulator of the respiratory metabolism [40]. Both the *SDH* gene (Figure 3) and SDH activity (Figure 1) were promoted after sand priming, which suggested that seed respiration was strengthened. Interestingly, the reduction of ROS would increase the stress tolerance. Our findings revealed a regulatory mechanism (Figure 4) related to the improvement of sand-priming treatment to seed germination of *Pinus massoniana*. Sand priming might modulate seed respiration, endogenous hormone and ROS content through affecting the interrelated genes expression, which could facilitate seed germination of *Pinus massoniana* conjointly.

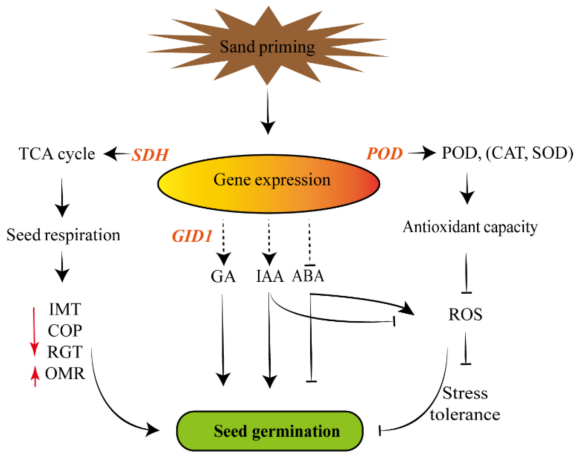

Figure 4. The regulatory mechanism of promotion of sand priming to seed germination in *Pinus massoniana*. Sand priming might enhance the relevant gene expression (*GID1*, *POD*, *SDH*, etc.). These genes might further affect the endogenous hormones level, antioxidant capacity and seed respiration. The enhancement of GA, IAA, low ROS and ABA might contribute to seed germination of *Pinus massoniana*. Simultaneously, it would enhance the stress tolerance, but it should be verified in follow-up research. SDH: succinate dehydrogenase; POD: peroxidase; CAT: catalase; SOD: superoxide dismutase; TCA: tricarboxylic acid; GID1: Gibberellin metabolism protein gene; GA: gibberellin; IAA: indole-3-acetic acid; ABA: abscisic acid; IMT: increased metabolism time; OMR: oxygen metabolism rate; COP: critical oxygen pressure; RGT: relative germination time; ROS: reactive oxygen species.

5. Conclusions

In conclusion, our results show that sand priming can promote the seed germination and field emergence, antioxidant ability and seed respiration of *Pinus massoniana*. Significant differences in germination rate, mean germination time, the activity of antioxidant enzymes (POD, SOD and CAT), endogenous phytohormone level (GA, IAA and ABA) and seed respiration were observed after sand-priming treatment. Sand as a solid matrix to prime the seeds of *Pinus massoniana* may contribute to breaking seed dormancy and improving the seed vitality of *Pinus massoniana* on a deeper layer, which will also provide an effective pretreatment method for the afforestation project.

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