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Metabonomics and physiology revealed the critical function of 5-Phosphoribosylamine and antioxidant enzymes in enhancing aged oat seed germination

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Abstract

Effective Microorganism (EM) is widely employed as a growth promoter in agricultural practices. The aging of oat seeds not only directly impairs agricultural production but also exerts adverse effects on biodiversity. The mechanism through which EM influence the germination of aging seeds remains unclear. In this experiment, the EM bacterial solution underwent pretreatment, which included the original-solution treatment (OrT), supernatant treatment (SuT), and sterile treatment (StT). Aging of oat seeds was induced using the pretreated EM bacterial solution. In this study, the EM bacterial solution facilitated the enhancement of the germination rate, germination index, and vitality index of aged seeds, with SuT demonstrating the most pronounced effects. Specifically, SuT resulted in a significant increase in APX and POD activities, while significantly reducing the malondialdehyde content. In addition, metabolic profiling highlighted the significance of 5-phosphoribosylamine in the purine metabolic pathway. Particularly in the SuT, the upregulation of 5-phosphoribosylamine facilitated the synthesis of (R)-Allantoin, consequently augmenting antioxidant enzyme activity.

Keywords Effective microorganisms, Age seeds, Seed germination, Metabolomics, Antioxidant enzymes, 5-Phosphoribosylamine

Introduction

Effective Microorganisms (EM) is a composite microbial agent comprising over 80 beneficial bacteria from 10 genera, including lactic acid bacteria, photosynthetic bacteria, and actinomycetes. Widely recognized for its effectiveness, EM is extensively employed as a growth promoter in agriculture [1, 2]. These microbial agents play a pivotal role in enhancing soil fertility, promoting plant growth, and consequently contributing to overall crop yield [3, 4]. For instance, EM microbial agents facilitate the introduction of a plethora of beneficial bacteria into the soil. The actions of these bacteria can augment

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the solubility of nutrients within the soil, reverse soil oxidation to an antioxidant state, and catalyze the activation of inorganic elements present in the soil. Consequently, this process enhances the absorption rate of crops [5]. Furthermore, EM agents also contribute to inhibiting harmful pathogens and pests while concurrently reducing the reliance on chemical pesticides [6]. In practical application, whether applied to rice, vegetables, fruits, or ornamental plants, EM agents consistently demonstrate a significant increase in yields [7–9]. The utilization of EM inoculants has notably enhanced the resilience of plants against diseases and adverse environmental conditions Dourado discovered that the application of EM and their metabolites could significantly decrease the incidence of plant pathogenic fungi in corn seeds, reducing the rate from 67 to 21%. Additionally, the study isolated non-pathogenic microorganisms from treated corn seeds and assessed their interactions with Fusarium in paired cultures. Among the 42 strains isolated from EM-treated seeds, 21 demonstrated the ability to inhibit Fusarium hyphal growth through antagonistic mechanisms, competition, and antimicrobial effects [10]. This improvement has led to significant enhancements in agricultural production [11, 12]. Given these benefits, EM inoculants have garnered widespread adoption in organic agriculture practices, thereby providing robust support for achieving more sustainable and environmentally friendly agricultural methods [7, 13]. Certainly, improper utilization of various types of EM extracts may sometimes result in failure to enhance crop yield and soil quality. Additionally, it can lead to a decrease in soil organic carbon and water-stable aggregates, which is detrimental to agricultural production [14, 15]. Therefore, it is imperative to conduct thorough and comprehensive research to elucidate the growth-promoting mechanism of EM bacterial solution. Consequently, three distinct treatment methods utilizing EM bacterial solution were employed in this experiment. In addition to the EM bacterial stock solution, the liquid was also subjected to centrifugation to isolate the supernatant, which is enriched with microbial metabolites, metabolic enzymes, and extracellular products. Furthermore, another group implemented a method to deactivate the EM bacterial fluid, thereby attenuating the influence of biological activity. These approaches aimed to investigate the effects of metabolites and microbial activity present in EM bacterial solution.

Despite the widespread usage and research on EM inoculants in various agricultural aspects, our understanding of their impact on seed germination, particularly on aged seeds, remains limited. Seed aging typically results in a diminished germination rate. Even if aged seeds do germinate, the ensuing seedlings often exhibit growth limitations, including slower growth, inadequate root development, and decreased resistance to pests and diseases [16, 17]. Aging seeds not only diminish production but also escalate planting costs [18]. Aged seeds exhibit lower and uneven germination rates, necessitating the use of more seeds to achieve the desired planting density [19]. This not only increases seed costs but also results in inconsistent crop growth, requiring additional labor and resources for management. To mitigate the low yields and address pest and disease issues associated with aged seeds, farmers often resort to increased use of pesticides and fertilizers [20]. Consequently, this practice can lead to soil and water pollution [21]. Therefore, Seed aging not only directly harms agricultural production but also has the potential to negatively impact the ecological environment and biodiversity [22]. Seeds possessing specific rare or unique genetic traits may lose their germination ability as they age, leading to the potential loss of invaluable genetic resources [23]. Enhancing the germination rate of aged seeds holds not only immediate economic and practical value for agricultural production but also carries profound implications for the environment, ecology, and scientific research. Hence, we posit a new hypothesis suggesting that EM may possess the capability to promote the germination of aged seeds. If this hypothesis is confirmed, it could present a novel strategy for agricultural production, particularly in enhancing the germination efficiency of aged seeds. This could lead to increased planting efficiency and income for farmers. The vitality and quality of seeds represent a pivotal aspect of global food production and agricultural sustainability [24]. Seeds are susceptible to aging during storage, particularly in environments characterized by high temperatures and humidity [23]. This aging process encompasses various morphological, physiological, and biochemical changes, including membrane damage, structural deterioration of organelles, loss of seed leachate, reduction in respiratory rate and ATP production, and enzyme inactivation [25]. More significantly, the excessive generation of reactive oxygen species (ROS) serves as a primary contributor to seed aging, leading to severe oxidative damage to cellular components such as proteins, lipids, chromosomal abnormalities, and DNA lesions [26]. Among these factors, mitochondrial dysfunction emerges as a key element in seed aging, with the excessive accumulation of ROS in mitochondria playing a decisive role [27]. During seed aging, degradation of cell membrane phospholipids, disruption of protein and genetic material structure and function, and attenuation of antioxidant system enzyme activity are observed [28]. In essence, seed aging is a multifaceted process encompassing a multitude of internal biochemical mechanisms and external environmental factors [24, 29].

Oat (*Avena sativa* L.) is recognized as an environmentally sustainable crop, cultivated extensively worldwide for its remarkable ability to thrive under diverse

environmental stresses [30]. However, oat grains tend to exhibit a high fat content, rendering them susceptible to rancidity or deterioration [31]. The oat variety "Qinghai 444 (QH444)" is extensively cultivated in China owing to its robust fibrous roots, vigorous growth, and resilience against lodging, drought, barrenness, and verticillium wilt. Therefore, in this study, we utilized seeds from the QH444 oat variety as materials for artificial aging treatment. Seeds age more rapidly when exposed to high temperatures (41 °C to 55 °C) and extremely high humidity (100% humidity) [32–34]. Subsequently, we employed an EM bacteria diluent as an initiator to revitalize the vigor of aging seeds. Our study aims to uncover the specific mechanisms responsible for the anti-aging properties of oat seeds and to evaluate the effectiveness of EM in this context. We investigate these mechanisms by analyzing the growth, physiological, and metabolic indicators of oat seeds. By examining growth dynamics, physiological changes, and metabolic activities, we seek to elucidate the mechanisms underlying the anti-aging characteristics of oat seeds. This research will provide valuable insights for advancing agricultural production and the seed industry.

Materials and methods

Test materials

The experiment was conducted at the Pratacultural Laboratory of Northeast Agricultural University. The test seeds utilized in the study were Qinghai No. 444 oat seeds, sourced from the Pratacultural Laboratory of Northeast Agricultural University. Mature and fully developed oat seeds were carefully chosen for the study. The EM bacterial solution is prepared using three distinct methods, denoted as OrT (original-solution treatment), SuT (supernatant treatment), and StT (sterile treatment). The specific processing methods for each treatment are outlined in Table 1.

Experimental design and process

Seed aging test

First, the seeds underwent an artificial aging process. Thirty seeds were placed in a glass Petri dish and incubated in a chamber set to 45 °C with 100% humidity. The artificial aging treatments were applied for durations of 24, 48, 72, 96, 120, and 144 h, with a control group of 0 h. Each treatment condition was conducted in triplicate. After the aging treatment, the seeds are transferred to the incubator (constant temperature 25℃, relative humidity 80%) in the absence of light. Germination indices were assessed and samples were collected upon seed germination (3 days). Meanwhile, the unsampled seeds were left to germinate unabated until the 7th day, with the daily growth data meticulously documented.

Seeds induced by EM bacteria liquid

For this stage, a predetermined number of seeds aged for 144 h were selected, following the seed aging method described in Sect. 2.2.1. Subsequently, the seeds were subjected to treatment with three different EM bacteria diluents (OrT, SuT, StT) applied by spraying onto their surface, while distilled water (H_2OT) served as the control. Each treatment condition was conducted in triplicate. Following this treatment, the seeds underwent a 4-hour period in an oven set at 35 °C. Afterward, they were removed and infiltrated with distilled water before being transferred to the incubator (constant temperature 25℃, relative humidity 80%) for a period of 7 days. Upon the germination of seeds (3 days post-treatment), germination indices were meticulously assessed, and samples were collected for further analysis. These samples were promptly frozen using liquid nitrogen and stored in a refrigerator at -80 °C for subsequent analysis. Meanwhile, unsampled seeds were allowed to germinate uninterrupted until the 7th day, during which daily growth data were meticulously documented. A portion of the frozen samples was allocated for the measurement of physiological indicators, while the remaining portion was forwarded to Hangzhou Lianchuan Biological Co., Ltd. for metabolomic analysis.

Determination of seed germination index

The procedures for measuring seed germination rate, germination index, and vigor index adhere to the guidelines established by the ISTA (International Seed Testing Association) [35]. Seed germination rate, germination index, and vitality index are calculated according to the following formula:

$$\text{Germination rate}\,(\%) = \frac{\text{total germination number}}{\text{test seed number}} \times 100\%$$

$$\text{Germination index} = \sum \frac{\text{germination number}}{\text{germination days}}$$

$$\text{Vigor index} = \text{germination index} \times (\text{root length} + \text{seedling length})$$

Table 1 Detailed information on the treatment method of EM microbial liquid

Treatment	EM bacterial solution processing method
OrT	Prepare the EM bacterial solution with a concentration of 0.5% (v/v) using distilled water
SuT	After centrifuging the EM bacterial solution, collect the supernatant and prepare it to a concentration of 0.5% (v/v) supernatant
StT	After subjecting the EM bacterial solution to high temperature (120℃, 2 h), prepare a solution with a concentration of 0.5% (v/v) using distilled water."

Determination of physiological indicators of seeds

Hydrogen peroxide (H_2O_2)

To quantify the H_2O_2 content in oat seeds post-priming with OrT, SuT, and StT, Solarbio's kit was employed. Initially, 0.1 g of oat seed samples were accurately weighed and ground into a fine powder using liquid nitrogen. Subsequently, the provided reagents were added to the samples in accordance with the instructions provided by Solarbio. Following the addition of reagents, the sample-reagent mixtures were thoroughly blended and allowed to incubate for the prescribed duration, as outlined in the kit instructions. Subsequently, the absorbance values of the samples were measured at a wavelength of 415 nm using a K6600-A microplate reader. Prior to measurement, the microplate reader was calibrated to ensure precise readings. To calculate the H_2O_2 content in the oat seed samples, the formula provided in the kit instructions was applied.

Superoxide anion (O_2^-)

The preparation of the standard curve, along with the measurement and calculation methods for O_2^-, is based on the work of Elstner and Heupel [36]. Accurately weigh a 0.1 g sample of oat seeds and grind it into a fine powder using liquid nitrogen. Next, prepare three test tubes for the sample analysis. Add 0.5 mL of the sample supernatant, 0.375 mL of phosphate buffer, and 0.125 mL of hydroxylamine hydrochloride to each tube. Incubate the tubes in a 25 °C water bath for 20 min. Then, add the reagents as done for the standard curve preparation and measure the absorbance at 530 nm.

Malondialdehyde (MDA)

The measurement and calculation of MDA in oat seeds treated with OrT, SuT, and StT follow the method outlined by Schmedes and Hølmer [37]. Weigh 0.1 g of the sample and add 1 mL of pre-cooled 10% trichloroacetic acid. Centrifuge the mixture at 4000 rpm for 10 min. Transfer 0.2 mL of the supernatant to a new tube. To this, add 1.8 mL of distilled water and 0.6% thiobarbituric acid solution. Mix thoroughly and incubate in a boiling water bath for 15 min. After incubation, cool the mixture rapidly. Take 200 μL of the cooled solution from each treatment and measure the absorbance at 600, 532, and 450 nm.

Proline (PRO)

The preparation of the standard curve, along with the measurement and calculation methods for proline, is based on the work of Ábrahám et al. [38]. Weigh a 0.1 g sample and grind it with liquid nitrogen. Plot the standard curve with proline content on the horizontal axis and absorbance on the vertical axis. Add 2 mL of glacial acetic acid, 2 mL of ninhydrin, and 1.5 mL of distilled water to 0.5 mL of the sample solution. For the control tube, add 2 mL each of glacial acetic acid, ninhydrin, and distilled water. Mix thoroughly. Develop the color and extract in the same manner as described for the standard curve preparation. Measure the absorbance at a wavelength of 520 nm.

Antioxidant enzyme activity

To measure Catalase (CAT), Superoxide dismutase (SOD), Peroxidase (POD) and Ascorbate peroxidase (APX), we utilized the kit provided by Solarbio, adhering strictly to the accompanying instructions. The absorbance of the specified band was measured using the K6600-A microplate reader, and calculations were performed according to the provided formula. For further details, please refer to the instructions available on the Solarbio website: https://www.solarbio.com/.

Metabolomics detection of oat seeds under different treatments

Based on the germination index and physiological index of seeds, SuT and H_2OT were chosen for metabonomic analysis, while samples without any initiation and aging treatment were designated as the control (CK). Three biological replicates were established in each group, and subsequently, the samples were sent to Hangzhou Lianchuan Biological Co., Ltd for subsequent metabonomic analysis.

Data analysis

The significance of differences between groups was analyzed using SPSS 26.0. LSD was chosen for post-hoc testing, with a significance level set at $P<0.05$. Line charts and bar charts were created using GraphPad Prism 9. Advanced volcano mapping was conducted using the OmicStudio tool (https://www.omicstudio.cn/tool). Additionally, a high-level pie chart illustrating the number of common differential metabolites between samples was generated using the OmicStudio tool. Metabolic pathway enrichment analysis was performed using MBRole 2.0, and the results were downloaded from http://csbg.cnb.csic.es/mbrole2/index.php. Subsequently, the OmicStudio tool was utilized to draw the KEGG enrichment pathway map. RDA analysis and Spearman correlation analysis between differential metabolites and physiological indicators were carried out using OmicStudio tools and visualized accordingly. The schematic diagram is drawn by figdraw (https://www.figdraw.com/).

Results and analysis

Effect of aging on germination of oat seeds

As illustrated in Fig. 1, subsequent to the aging treatment, there was a significant decline observed in the germination rate, germination index, and vigor index

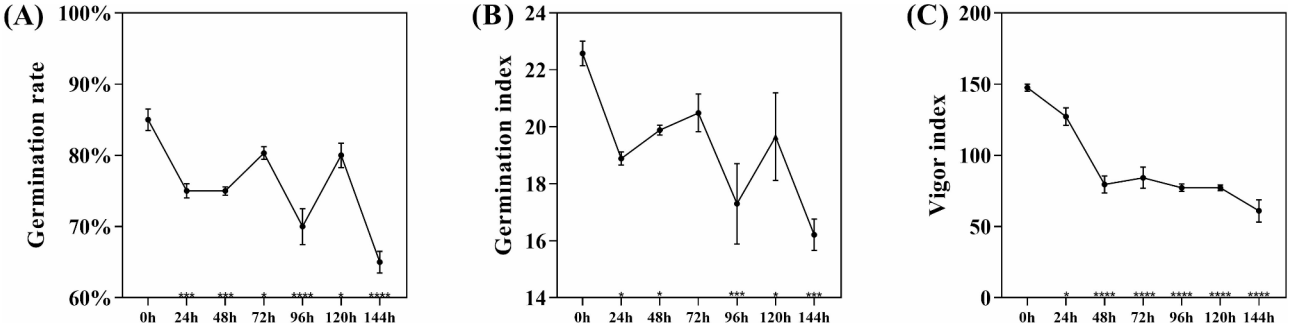

Fig. 1 The impact of artificial aging duration on oat seed germination. (**A**) The variation in oat seed germination rate with different artificial aging time; (**B**) The variation in oat seed germination index with different artificial aging time; (**C**) The variation in oat seed vigor index with different artificial aging time. The '*' symbol denotes significant differences between various processing times and 0 h (CK). (0.01≤*P<0.05, 0.001≤**P<0.01,0.0001≤***P<0.001, ****P<0.0001)

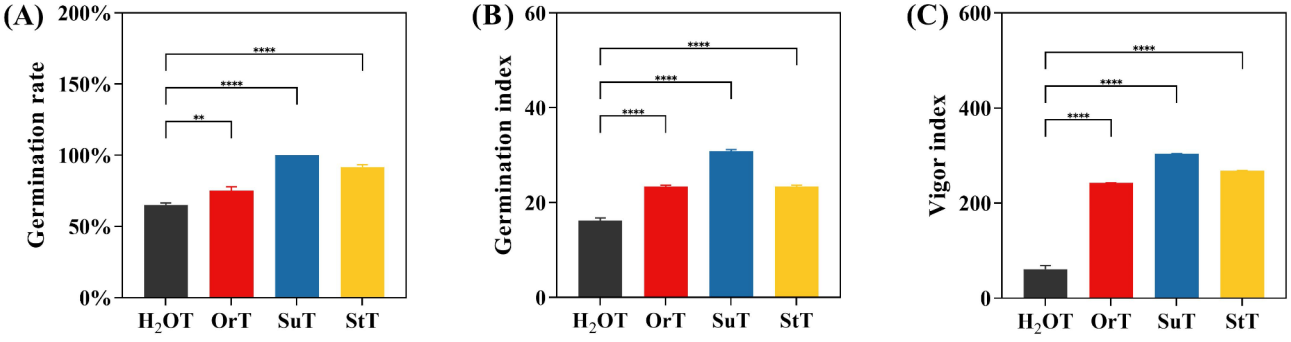

Fig. 2 The impact of EM bacterial solution on aged oat seed germination. (**A**) The variation in oat seed germination rate with different types of EM bacterial solution; (**B**) The variation in oat seed germination index with different types of EM bacterial solution; (**C**) The variation in oat seed vigor index with different types of EM bacterial solution. The '*' symbol denotes significant differences between different types of EM bacterial solution treatments and the H_2OT (CK), The same notation applies below for subsequent analyses. (0.01≤*P<0.05, 0.001≤**P<0.01,0.0001≤***P<0.001, ****P<0.0001)

of oat seeds, correlating with the duration of aging. Following exposure to a 144-hour aging treatment, the oat seeds exhibited a reduction of 23.53% in the germination rate, a decrease of 28.21% in the germination index, and a decline of 58.63% in the vigor index.

Effect of EM bacterial solution on germination of aged oat seeds

Figure 2 depicts the influence of EM bacterial solution on the germination rate, germination index, and vigor index of aged seeds. Significantly, OrT demonstrated a notable increase in the seed germination rate by 15.38%, the germination index by 30.71%, and the vigor index by 296.38%. Similarly, SuT exhibited a remarkable increase in the seed germination rate by 53.85%, the germination index by 90.36%, and the vigor index by 397.29%. StT displayed a noteworthy increase in the seed germination rate by 41.03%, the germination index by 44.31%, and the vigor index by 339.12%.

Effects of EM bacterial solution on physiology of aged oat seeds

Figure 3 illustrates the impact of EM bacterial solution on oat seed physiology. Notably, OrT demonstrated a considerable decrease in the MDA content by 68.33% and a significant increase in APX activity by 69.88%. SuT resulted in a significant reduction in MDA content by 50.00%, a notable increase in POD activity by 68.25%, and a significant elevation in APX activity by 94.31%. Additionally, StT exhibited significant reductions in MDA content by 41.67% and H_2O_2 content by 42.28%, alongside a notable decrease in POD activity by 82.28%.

KEGG enrichment analysis of differential metabolites

Figure 4 provides comprehensive details regarding the differential metabolites observed across various comparison groups. Specifically, a total of 122 differential metabolites were identified between the H_2OT and CK comparison groups, consisting of 64 up-regulated and 58 down-regulated metabolites (Fig. 4A). These metabolites are associated with 9 pathways, with Histidine metabolism being the most significantly enriched pathway (Fig. 4D). In the comparison group of SuT and CK, a total of 381 differential metabolites were identified, with 260 up-regulated and 121 down-regulated metabolites (Fig. 4B). These metabolites are associated with 51 pathways, with the Aminobenzoate degradation pathway being the most significantly enriched (Fig. 4D).

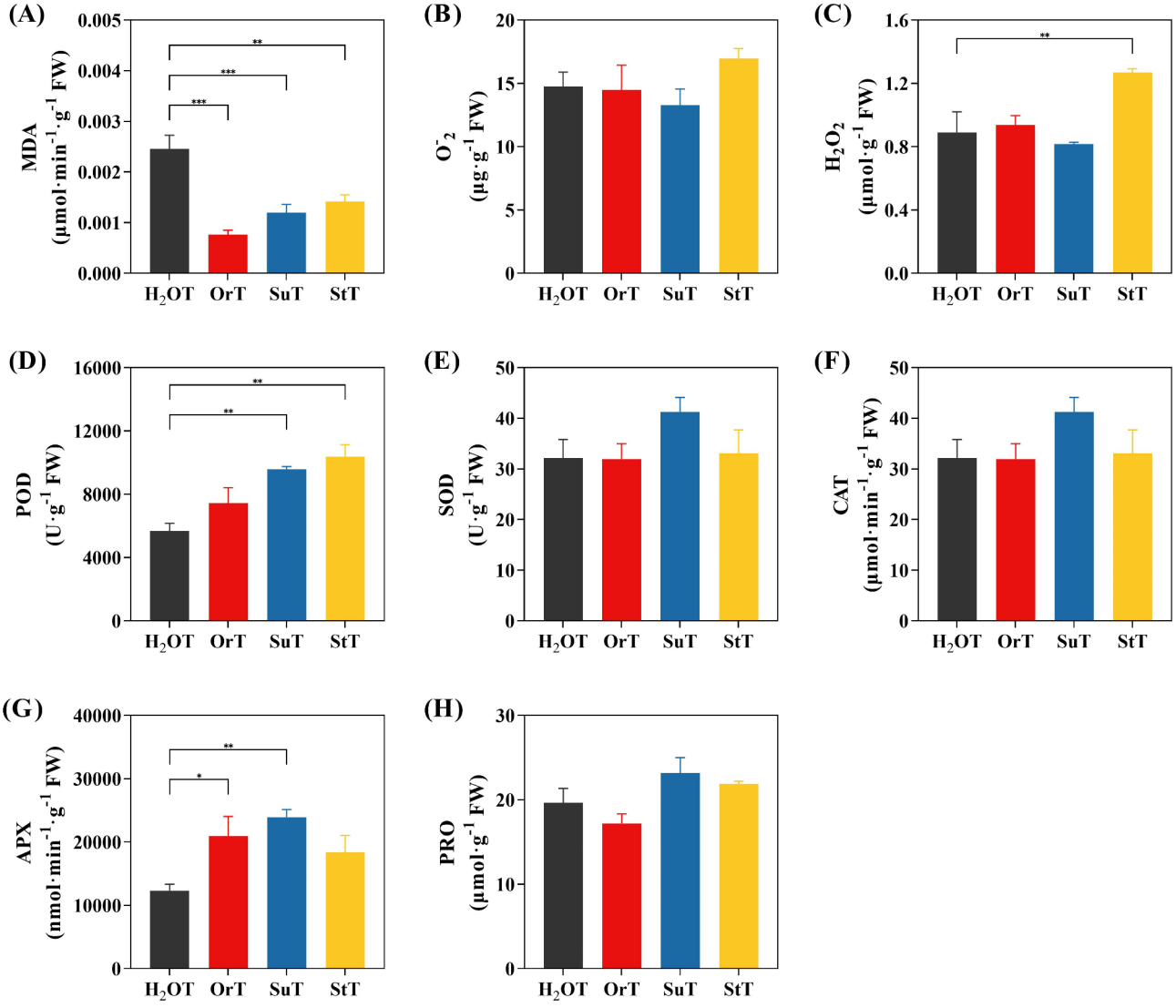

Fig. 3 The impact of EM bacterial solution on aged oat seed physiology. (**A**) The variation in oat seed MDA content with different types of EM bacterial solution; (**B**) The variation in oat seed O_2^- content with different types of EM bacterial solution; (**C**) The variation in oat seed H_2O_2 content with different types of EM bacterial solution; (**D**) The variation in oat seed POD activity with different types of EM bacterial solution; (**E**) The variation in oat seed SOD activity with different types of EM bacterial solution; (**F**) The variation in oat seed CAT activity with different types of EM bacterial solution; (**G**) The variation in oat seed APX activity with different types of EM bacterial solution; (**H**) The variation in oat seed PRO content with different types of EM bacterial solution. The '*' symbol denotes significant differences between different types of EM bacterial solution treatments and the H_2OT (CK). ($0.01 \leq {}^{*}P < 0.05$, $0.001 \leq {}^{**}P < 0.01$, $0.0001 \leq {}^{***}P < 0.001$, ${}^{****}P < 0.0001$)

Furthermore, it is noteworthy that 11 differential metabolites were consistently identified across all comparison groups (Fig. 4C).

Identification of key differential metabolites

Physiological indicators of oat seeds were utilized as response variables, with significantly different metabolites from the three treatments selected as explanatory variables. Redundancy analysis unveiled the metabolites influencing physiological indicators of purple oat seeds. As depicted in Fig. 5A, the cumulative explained variation of the first and second axes reached 79.52%, effectively reflecting the impact of significantly different metabolites on physiological indicators. Notably, 5-phosphoribosylamine exhibited robust explanatory power on physiological indicators, with an r^2 value of 0.8585.

Furthermore, Spearman correlation analysis was conducted on significantly different metabolites and physiological indicators among the three treatments, as shown in Fig. 5B. The findings revealed that differential metabolites significantly influenced oat seed physiological indicators. Specifically, seven metabolites exhibited significant correlations with physiological indicators ($rho > 0.8$, $P < 0.05$). Notably, MDA

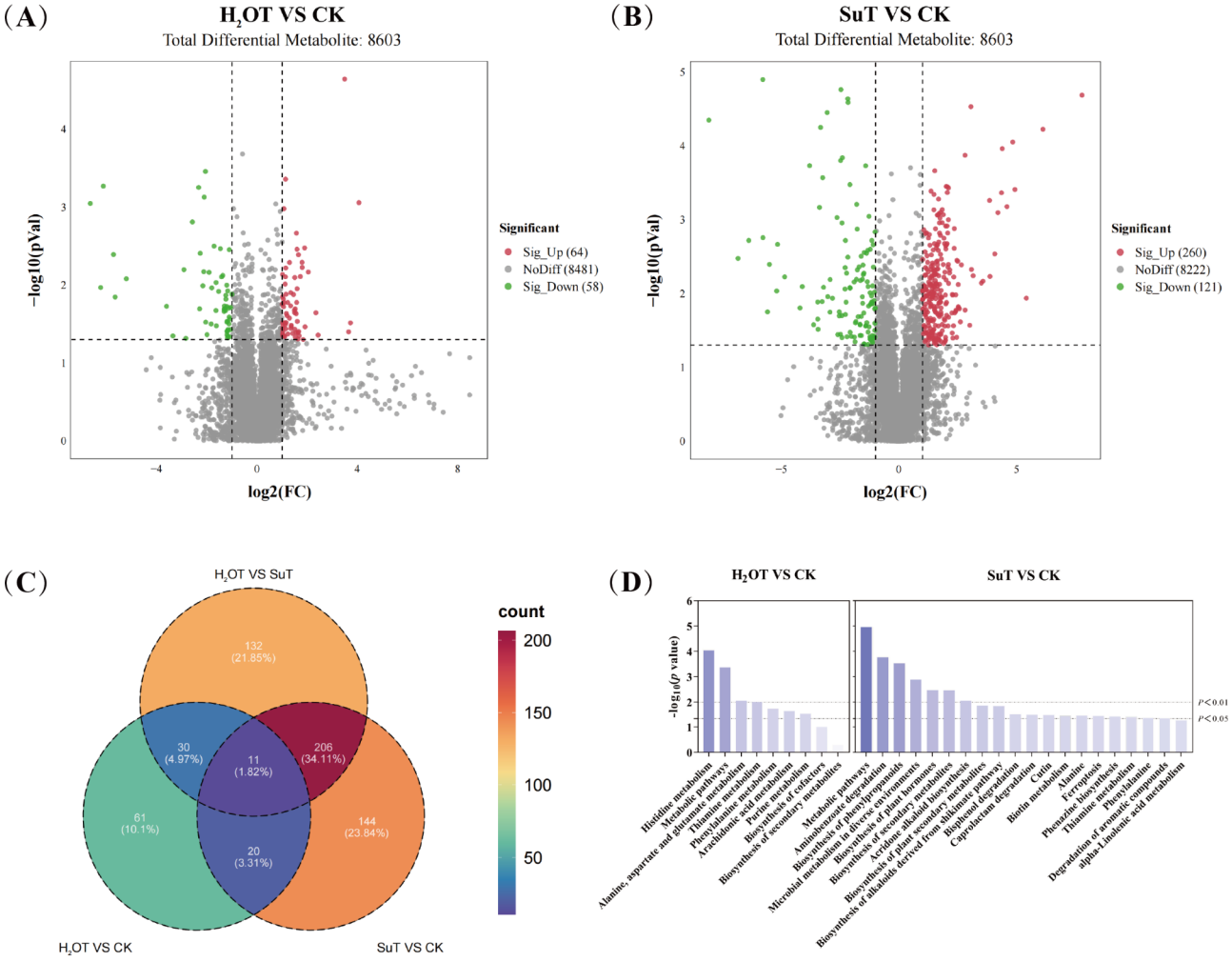

Fig. 4 Analysis of differential metabolites and KEGG function between comparison groups. (**A**) Volcano diagram of differential metabolites between H_2OT and CK ; (**B**) Volcano plot of differential metabolites between SuT and CK ; (**C**) Wayne diagram of differential metabolites between different treatments ; (**D**) KEGG enrichment pathway of differential metabolites

demonstrated significant negative correlations with four differential metabolites, namely neg-3.266_145.05147, neg-3.267_135.06708, neg-3.267_147.03088, and 5-phosphoribosylamine. In this study, 5-phosphoribosylamine emerged as prominently associated with seed physiology in both statistical analyses. Subsequently, this experiment delves into investigating the specific role of 5-phosphoribosylamine (5-PRA) within metabolic pathways, building upon prior research.

Effect of EM bacterial solution on metabolic pathways of aged seeds

Figure 6 illustrates the principal metabolic pathways associated with 5-PRA. These pathways include Alanine, aspartate, and glutamate metabolism, as well as Purine metabolism. Interestingly, no significant alterations in metabolite levels were detected in Alanine, aspartate, and glutamate metabolism, with 5-PRA primarily deriving from the breakdown of L-Glutamine. In Purine metabolism, 5-PRA serves as a crucial intermediate, displaying significantly heightened levels under SuT conditions. Guanine and Adenine, both products of Purine metabolism, exhibit decreased levels in H_2OT and SuT compared to CK. Furthermore, (R)-Allantoin, another product of Purine metabolism, demonstrates markedly increased levels in SuT compared to H_2OT and CK. Additionally, there is a noteworthy rise in the content of the (R)-Allantoin synthetic precursor, 5-hydroxyisourate.

Discussion

Senescence is thought to delay germination and seedling emergence, hinder growth rates, and increase sensitivity to environmental stress. It can also lead to higher levels of genomic damage, which compromises germination potential, induces instability in the plant genome, and impedes overall growth [17, 39]. In this study, following the exposure of seeds to 144 h of artificial aging, notable decreases were observed in the germination

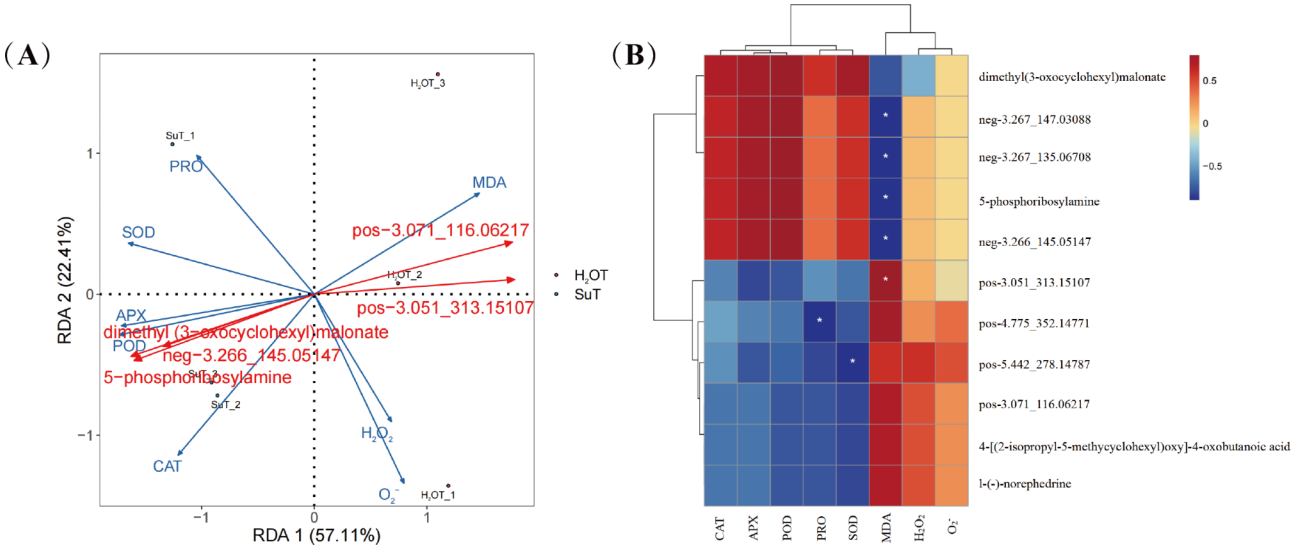

Fig. 5 Correlation between differential metabolites and physiology of oat seeds. (**A**) Redundancy analysis of differential metabolites and oat seed physiology in a single sample of different treatments. The blue arrow represents the physiological index of oat seeds, the red arrow represents the differential metabolites, and the length of the arrow line represents the effect of differential metabolites on the physiology of oat seeds. The longer the arrow length, the greater the effect. (**B**) Spearman correlation between 11 common differential metabolites and physiological indexes of oat seeds, *0.01 ≤ *P*<0.05

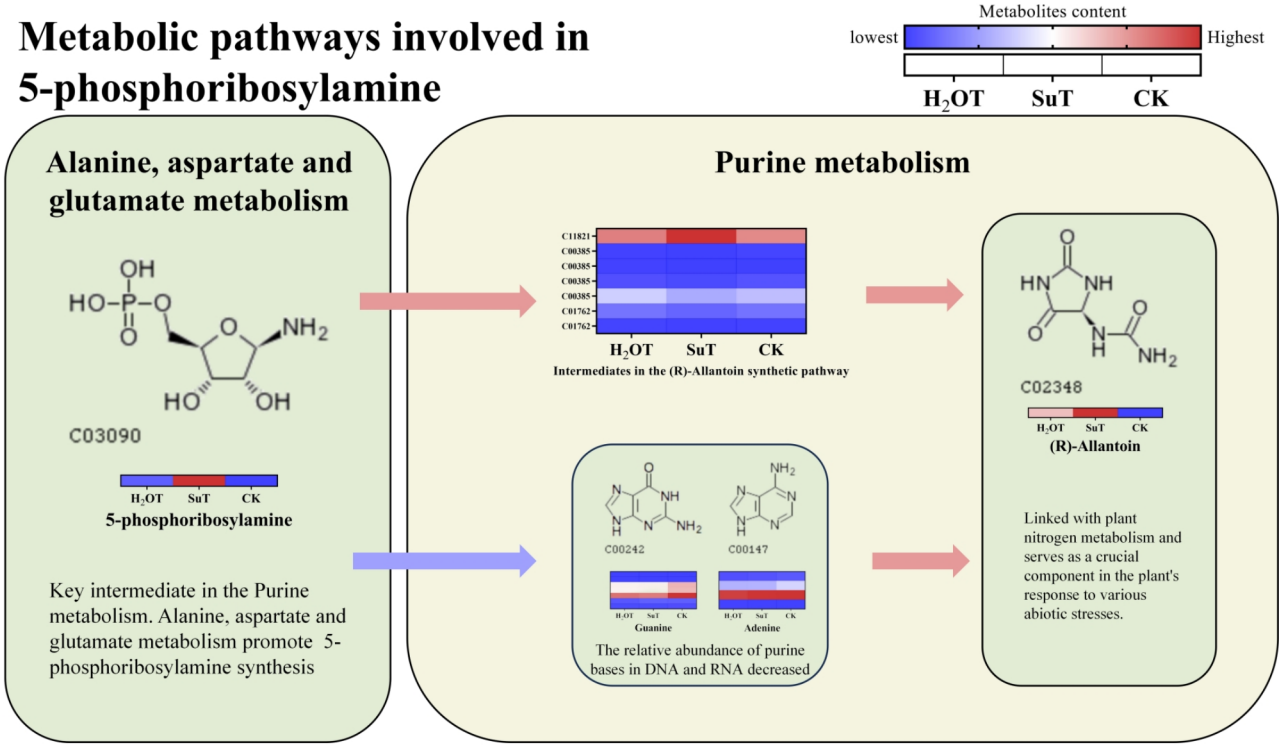

Fig. 6 Changes in the main metabolic pathways in aged oat seeds after induced by SuT

rate, germination index, and vigor index, with the most significant decline noted in the vigor index. Furthermore, the utilization of EM bacterial solution notably augmented the germination rate, germination index, and vigor index of aged oat seeds. Particularly noteworthy was the remarkable effectiveness of the SuT in alleviating the detrimental effects of seed aging. Shaffique's research demonstrates that Bacillus enhances the germination rate, germination index, and vigor index of rice seeds [40]. This finding underscores the capacity of certain microorganisms to promote seed germination, which aligns with the results obtained in this study. The

application of three different EM bacterial extract treatments significantly enhances the vigor index of aged oat seeds. Enhancing seed vigor index is crucial for promoting the healthy growth of plant seedlings [41]. Seeds with high vigor possess the ability to germinate and develop at an accelerated rate, enabling crops to establish robust root and plant systems during the early stages of growth [42]. This capability aids in minimizing crop losses attributable to external environmental factors. OrT, SuT, and StT extracts all exhibit the ability to promote seed germination, albeit with varying effects. This indicates that the mere activity of microorganisms may not be the primary determinant in facilitating seed germination. Instead, microbial metabolites are likely to play a crucial role. Specifically, the presence of active microbial metabolites and an abundance of metabolic enzymes within the SuT extract may serve as key factors in promoting the germination of aged oat seeds. It is possible that in SuT, bacteria and larger cell debris are separated and deposited at the bottom of the tube due to centrifugal force, leading to the release of their metabolites into the supernatant as a result of cell rupture. This process could potentially increase the presence of metabolites beneficial for anti-aging effects compared to OrT. Conversely, subjecting StT to high temperatures may result in the inactivation of certain metabolites [43].

The enhancement of seed germination indicators may arise from improvements in seed physiology. In this study, OrT, SuT, and StT significantly reduced the MDA content of oat seeds. A study conducted by Li et al. demonstrated a similar effect, where immersion of maize seeds in suspension of strain HX-2 resulted in a decrease of 42.38% in MDA content, indicating a positive effect of bacterial treatment on reducing MDA levels in seeds [44]. The decrease in malondialdehyde production can be attributed to the impact of microbial metabolites present in EM solutions on plant metabolic processes. This phenomenon likely involves the modulation of redox equilibrium within plants and the enhancement of antioxidant substance synthesis [45]. Reactive oxygen species can induce oxidative stress in seeds; however, this stress can be mitigated by the action of antioxidant enzymes [46, 47]. In this study, the H_2O_2 content in StT increased significantly, but this was not accompanied by an increase in MDA. In contrast, the study by Yao et al. found a strong correlation between H_2O_2 and MDA, which differs from the findings in this study [48]. This discrepancy may be attributed to an increase in H_2O_2 induced by cellular components or PAMPs produced during high-temperature inactivation [49]. Additionally, the changes in MDA content may be attributed to the presence of active metabolites in the EM bacterial solution, which can modulate the levels of H2O2 and MDA by influencing the activity of antioxidant enzymes [50, 51]. The successful germination of seeds hinges upon maintaining a delicate balance between the rate of ROS accumulation and the activity of the ROS neutralization system. These roles include weakening the endosperm, mobilizing seed reserves, providing defense against pathogens, and facilitating programmed cell death. Moreover, ROS can serve as messengers or transmitters of environmental signals during seed germination [52]. In this study, it was observed that the levels of O_2^- and H_2O_2 in SuT samples decreased slightly, although not significantly, and the overall levels of active oxygen did not undergo drastic changes. This suggests that the SuT bacterial solution induces a more gradual alteration in active oxygen levels, which may potentially be more advantageous for seed germination. Conversely, the significant increase in H_2O_2 content observed in StT indicates that microbial activity plays a crucial role in regulating and maintaining stable levels of reactive oxygen species. Additionally, all three EM bacterial solutions led to increased activities of POD and APX, further indicating their positive influence on antioxidant enzyme systems. In Shaffique's study, SH-9 was found to enhance the growth of rice plants, coinciding with a significant increase in the activities of antioxidant molecules including CAT, SOD, and APX. This observation is consistent with the findings of the current study [40]. The elevation in antioxidant enzyme activity may be attributed to microorganisms acting as a source of antioxidants [53, 54]. Moreover, microbial metabolites could potentially trigger specific regulatory mechanisms related to antioxidant enzymes [55]. The increase in APX activity signifies an enhanced ability of oat seeds to finely regulate the levels of reactive oxygen species [56]. POD enzymes are involved in plant differentiation and are also known to respond to environmental stress [57]. The observed highest POD activity in StT could be attributed to the fact that H_2O_2 serves as the substrate for POD. Therefore, a significant increase in substrate content leads to a notable increase in enzyme activity [58, 59]. Notably, the SuT significantly elevated POD and APX activities and exhibited the highest levels of SOD and CAT activities, although the differences were not statistically significant. SOD serves as the primary enzyme in the antioxidant system, responsible for catalyzing the dismutation of superoxide radicals into H_2O_2 [60]. CAT is primarily responsible for catalyzing the decomposition of H_2O_2 into water and oxygen [61]. The positive effect of SuT on the antioxidant enzyme activity of oat seeds helps maintain the stability of moderate levels of ROS, while simultaneously keeping the MDA content of oat seeds at a low level. This preservation of physiological quality in the seeds is more conducive to germination [60].

EM can enhance plant tolerance to various environmental stresses by inducing changes in metabolic pathways. Examples of these changes include the synthesis

of osmolytes, antioxidants, and stress-related hormones, all of which aid plants in coping with adverse conditions [62–64]. In this study, it was observed that SuT altered the pathway with the most significant enrichment of differential metabolites from Histidine metabolism to the aminobenzoate degradation pathway. Histidine serves as a precursor for the biosynthesis of various secondary metabolites in plants, including alkaloids, flavonoids, and cyanogenic glycosides [65]. These secondary metabolites play crucial roles in plant defense mechanisms, stress responses, and signaling pathways [66]. The enrichment of aminobenzoate degradation pathways may be attributed to EM. The abundance of bacteria within EM provides significant biodegradation capabilities [67]. Furthermore, plants and EM can utilize the metabolites resulting from aminobenzoate degradation as either carbon or energy sources [68]. Moreover, by facilitating the breakdown of organic pollutants such as aminobenzoates, this process can mitigate soil and water contamination, thereby safeguarding the health of the ecological environment [69].

5-PRA serves as a pivotal intermediate in an alternative initial pathway of purine biosynthesis, rendering it a crucial metabolite within this biosynthetic process [70]. In this study, the metabolites of 5-PRA that were consistently present across all comparison groups were found to exhibit a stronger correlation with seed physiology in statistical analyses. Consequently, these metabolites were identified as key differential metabolites. In the current investigation, a noteworthy elevation of 5-PRA was observed in the H_2OT treatment group, albeit not as pronounced as in the SuT group. This underscores the collective impact of microbial metabolites, metabolic enzymes, extracellular products, and the water component present in the EM bacterial supernatant, which contributes to the upregulation of 5-PRA. 5-PRA is likely produced through the Alanine, aspartate, and glutamate metabolism pathways, arising from the breakdown of L-Glutamine [71]. Notably, our experiment did not observe substantial alterations in other metabolites within the 5-PRA synthesis pathway, potentially attributed to feedback inhibition mechanisms [72]. Following its upregulation, 5-PRA entered purine metabolism; however, it did not induce notable changes in the levels of guanine and adenine, which are the other two key products in purine metabolism. Moreover, there were no significant alterations observed in other intermediate products of the guanine and adenine synthesis pathways. This suggests that the synthesis of guanine and adenine remains unaffected by the upregulation of 5-PRA. Conversely, within the synthesis pathway of (R)-Allantoin, another metabolite of Purine metabolism, a significant upregulation of (R)-Allantoin and its associated intermediates was evident. Irani and Christopher D Todd highlighted that *Arabidopsis thaliana* seedlings exhibiting elevated levels of allantoin demonstrate enhanced tolerance to drought and sodium chloride stress. The heightened concentrations of allantoin are believed to mitigate oxidative damage, thereby enhancing the plant's resilience to abiotic stress conditions in *Arabidopsis thaliana* [73]. In another study, it was observed that exogenous allantoin upregulated the expression of several antioxidant-encoding genes, such as SOS1 and RCD1 [74]. In this study, it was observed that SuT exhibited elevated antioxidant enzyme activity, a phenomenon that may be associated with the augmented levels of (R)-Allantoin. Hence, SuT likely induces a notable upregulation in the synthesis of (R)-Allantoin within Purine metabolism by facilitating the production of 5-PRA. Subsequently, (R)-Allantoin is presumed to augment the activities of key antioxidant enzymes such as POD, SOD, CAT, and APX by upregulating the expression of genes involved in oxidative stress response. This enhancement significantly boosts the antioxidant capacity of oat seeds, allowing for precise regulation of reactive oxygen species levels. Consequently, despite a considerable decrease in the MDA content of oat seeds, there is a steady decline in the concentration of reactive oxygen species. This consistent maintenance of cellular redox equilibrium ensures minimal fluctuations in cell redox states, thereby preserving the integrity of cellular processes and intracellular reactions [75]. Consequently, this state renders oat seeds more conducive to successful germination.

Future studies should focus on identifying the regulatory genes involved in 5-PRA synthesis and elucidating their regulatory mechanisms. Furthermore, there is an urgent need to clarify the specific components of microbial active metabolites in the EM solution that promote seed germination. This study also acknowledges certain limitations that may influence the interpretation of the results. While it demonstrated that effective microbial EM has beneficial effects on oat seeds, it did not confirm whether EM exhibits broad biological adaptability. Investigating this aspect is essential to uncover the underlying mechanisms of microbial growth and its potential applications in agricultural production.

Conclusion

The detrimental effects of oat seed aging intensify with prolonged exposure to aging conditions. Significant reductions in germination rate, germination index, and vigor index of oat seeds were observed following aging. However, treatment with EM bacteria solution demonstrated a capacity to restore the germination ability of oat seeds, with the most notable enhancement observed in seed vigor. This improvement in germination ability is attributed to decreased levels of malondialdehyde and enhanced antioxidant capacity within the seeds.

Moreover, analysis of three distinct EM treatments revealed that the active metabolites produced by EM bacteria played a pivotal role in promoting seed germination, rather than the microorganisms themselves. Metabolic profiling highlighted the significance of 5-phosphoribosylamine in the purine metabolic pathway. Particularly in the SuT, the upregulation of 5-phosphoribosylamine facilitated the synthesis of (R)-Allantoin, consequently augmenting antioxidant enzyme activity. The findings of this study not only offer a theoretical foundation for enhancing the quality of aged seeds but also present a promising approach for investigating the molecular responses of various plants to EM bacteria solution. This research provides valuable insights into strategies for improving seed quality.

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Author contributions
Conceptualization, data curation and formal analysis, Y.H. and L.D.; Investigation, L.D.; Methodology, Y.H.; Resources and software , S.S.; Supervision and validation, K.W.; Visualization, Y.Z.; Writing original draft, T.G.; Writing review & editing, Y.G.; Funding acquisition and project administration, G.H. and L.Q. All authors reviewed the manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
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