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Phenotypic, Physiological and Hormonal Analysis Reveals the Mechanisms of Timely Harvesting for Ensuring the Seed Vigor of Maize (*Zea mays* L.) Inbred Lines

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Abstract: Seed vigor is a pivotal indicator of seed quality, and timely harvesting is essential for maize seed vigor. The seeds and embryos of maize inbred lines JNY6F and PH4CV at different maturity stages were selected as study materials, the phenotypic characteristics and seed vigor indexes of which were detected, and the soluble sugars, antioxidant enzyme activity, and pythormones [auxin (IAA), cytokinins (CTKs), gibberellins (GAs), and abscisic acid (ABA)] in fresh immature embryos were analyzed. The analysis results indicated that the seeds of JNY6F and PH4CV reached physiological maturity at 35 and 50 days after pollination, which were the optimal harvest times for JNY6F and PH4CV, respectively, as the embryonic morphology of which had been estabilished, and the seed vigor of which reached their peaks at these two stages. The seed vigor indexes showed significant negative correlations with the levels of soluble reducing sugar, total soluble sugar, and four pythormones in the immature embryos, but were highly positively correlated with catalase (CAT) and peroxidase (POD) enzyme activities. In summary, our findings offer valuable insights into the ideal harvest time and physiological mechanisms underlying the seed vigor of maize inbred lines, and contribute to the enhancement of seed quality and agricultural practices in maize inbred line production.

Keywords: maize inbred lines; seed vigor; timely harvesting; phytohormone level

Citation: Feng, W.; Wang, H.; Bai, Y.; Wang, W.; Xue, W.; Shi, Z.; Qiu, P.; Gao, Y.; Xue, J.; Sun, M.; et al. Phenotypic, Physiological and Hormonal Analysis Reveals the Mechanisms of Timely Harvesting for Ensuring the Seed Vigor of Maize (*Zea mays* L.) Inbred Lines. *Agronomy* **2024**, *14*, 1770. https://doi.org/10.3390/agronomy14081770

Academic Editor: Enrico Porceddu

Received: 23 June 2024
Revised: 26 July 2024
Accepted: 7 August 2024
Published: 13 August 2024

1. Introduction

Maize (*Zea mays* L.) is the most essential crop worldwide for food, feed livestock and bioenergy production, and also an ideal model plant for genetic study [1]. In the past few decades, more than 95% of maize growing areas has been planted with hybrid seeds, due to their high yield and greater adaptability [2], and the annual production of maize has been exceeding 1 billion tons, establishing itself as a staple grain in many countries [3]. Presently, single-seed precise sowing of hybrid maize is gaining significant traction in China [4]. Consequently, seed and seedling quality should be of concern, as high seedling emergence rate is the most important aspect to ensure high yield [5]. Seed quality is generally reflected by seed vigor, which is a critical trait that determines the potential ability for successful germination and seedling establishment under various environmental conditions [6,7]. Highly vigorous seeds exhibit uniformity in germination and successful seedling establishment, ultimately improving crop productivity [8]. Hence, producing hybrid maize seeds with high seed vigor is indispensable for a seed company's economic benefits, as well as the powerful guarantee for Chinese food security [9].

Seed vigor is mainly tested by physiological and biochemical methods, which measure germination characteristics and specific biochemical reactions associated with seed vigor, respectively. The methods used for measuring seed vigor under controlled germination conditions are subsequently utilized to predict seed germination performance in fields. The standard germination (SG) test is typically employed to predict field emergence under optimal situations [10]. In addition, several indicators are used to evaluate seed vigor, such as germination rate (GR), germination energy (GE), germination index (GI), and seedling vigor index (SVI), which were recommended by the International Association for Seed Testing (ISTA) [11]. Prior studies have demonstrated that the activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) has been observed to be closely related to seed vigor [12]. Additionally, soluble sugars (SS) can mitigate desiccation damage by stabilizing proteins, membranes, and macromolecules during maturation, and enhance seed vigor and seedling growth by providing energy [13,14]. These findings underscore the complexity of assessing seed vigor; relying solely on a single test or vigor index to determine seed vigor may be inaccurate [15]. Thus, it is necessary to conduct a comprehensive approach involving multiple tests and biochemical assessments.

Endogenous plant hormones are pivotal regulators of seed dormancy and germination, and the hormone concentrations and seed sensitivity play essential roles in seed germination. Among them, abscisic acid (ABA) functions as a principal inducer and protector of seed dormancy, and excessive accumulation of ABA can lead to profound seed dormancy [16–18]. On the other hand, gibberellins (GA) can induce the expression of hydrolytic enzymes such as α-amylase, protease, nucleases, and phospholipases in the endosperm cells, hydrolyzing seed storage materials to provide energy and substrates for seed germination, thereby breaking the dormancy caused by high ABA levels [19–21]. Cytokinins (CTK) can antagonize ABA in seed germination, counteracting its inhibitory effect on germination [22,23]. CTK can also interact with GA to induce α-amylase expression, further facilitating seed germination [24–26]. Additionally, auxin (IAA) positively regulates ABA biosynthesis, and negatively regulates GA biosynthesis and signaling pathways during seed germination, thereby inhibiting seed germination [27,28].

Harvest time is widely acknowledged as a crucial factor in determining seed vigor [29,30]. Previous studies have identified the ideal harvest time (IHT) as the phase when seeds reach their maximum vigor, which typically occurs a few days prior to physiological maturity in maize seeds [31,32]. However, these studies are predominantly focused on the seed vigor of hybrid maize, with limited reports emphasizing the seed vigor of maize inbred lines. Maize inbred lines, developed through self-pollination, are valuable genetic resources extensively utilized in the development of hybrid varieties [33]. Therefore, the development and utilization of maize inbred lines have substantially contributed to the advancement of maize breeding programs. Above all, we proposed that assessing the dynamic changes of seed vigor of inbred lines at different days after pollination (DAP) may provide insights for the identification of maize lines with superior germination rates, and facilitate the selection of parental lines with complementary vigor traits, resulting in enhanced hybrid performance. In this study, the moisture content, dry matter accumulation rate, and seed vigor were analyzed, followed by an analysis of the changes in soluble sugar content antioxidant enzyme activities, and endogenous hormone content in fresh embryos at different maturity stages. The objective of this study was to elucidate the patterns of seed vigor variation in maize inbred lines and explore the underlying mechanisms, ultimately contributing to a better understanding of the mechanisms of seed vigor formation.

2. Materials and Methods

2.1. Material Planting and Collection

The maize inbred lines JNY6F (early maturity), selected from the progeny of hybrid maize Dika517 by us, and PH4CV (later maturity) were cultivated in the experimental field of Shanxi Agricultural University (112°34′ E, 37°25′ N) on 2 May 2020 and 4 May 2021, Shanxi, China. The experimental area is situated in a warm temperate continental

climate zone at an elevation of 791 m above sea level. Throughout the maize growth period, which spans from May to September, the total precipitation was 321.5 mm in 2020 and 313.9 mm in 2021, with average temperatures of 21.3 °C in 2020 and 21.7 °C in 2021. Prior to the experiment, the topsoil (0–20 cm) was sandy loam with a pH value of 7.78. Soil nutrient content before sowing was as follows: total nitrogen, 1.26 g kg^{-1}; alkaline hydrolyzable nitrogen, 84.28 mg kg^{-1}; available phosphorus, 26.38 mg kg^{-1}; available potassium, 124.65 mg kg^{-1}; and organic matter, 18.82 g kg^{-1}.

The experiment was conducted using a randomized complete block design (RCBD) and involving 2 maize inbred lines. Each inbred line was planted in 3 plots, with a length of 5 m, row spacing of 0.5 m, and a density of 60,000 ha^{-1}. Prior to sowing, 240 kg ha^{-1} of pure nitrogen (urea, 46% N), 120 kg ha^{-1} of pure phosphorus (12% P_2O_5), and 240 kg ha^{-1} of pure potassium (60% K_2O) were evenly applied. The field received irrigation at the jointing, booting, and anthesis-silking stages, with local measures taken for artificial weed control and pest and disease management. JNY6F and PH4CV were pollinated in early and middle July, respectively. All the individual plants for each inbred line were self-pollinated simultaneously. To minimize the impacts of different ear positions on the seed vigor, and the physiological and hormone biosynthesis changes in seeds [34,35], the uniform seeds and embryos of JNY6F from 20 days after pollination (DAP) to 50 DAP, and PH4CV from 20 DAP to 60 DAP, with a five-day intervals, were collected from the mid-part of the ears by manual dissection. Three biological replicates were performed for each of the time points. Each replicate was obtained by pooling samples from at least five ears. The replicates of each sample were used to carry out the phenotypic and physiological analysis and germination assay.

2.2. Phenotypic Analysis and Moisture Content Detemination

Representative seeds were selected for photography using a camera, and 100 to 200 seeds and embryos of the two maize inbred lines from each replicate were weighed firstly, then placed in the oven for 30 min at 120 °C, and dried at 80 °C until constant weight; thus, the dry weights and moisture content of those were analyzed according to the method of Hay et al. [36]. A modified paraffin section method was used for anatomical examination of the immature embryos [37]. Briefly, samples were removed from the FAA fixative, dehydrated through a graded tertiary butyl alcohol series, and embedded in paraffin. Tissue sections (10 μm) were cut longitudinally from the wax-embedded samples by a microtome, affixed to glass microscope slides, and stained with 0.05% toluidine blue for observation using a microscope (Olympus BX51).

The formula for calculating the moisture content of seeds and embryos is as follows [36]:

$$\text{MC } (\%) = \frac{\text{starting sample weight} - \text{dry weight}}{\text{starting sample weigh}} \times 100 \quad (1)$$

2.3. Germination Assay

Approximately 100 seeds were naturally dried and sterilized in 10% (v/v) H_2O_2 for 30 min, washed by distilled water twice and soaked for 12 h in distilled water, then transferred into Petri dishes with double moist filter papers, and germinated in the dark at 25 °C with a 12 h light/12 h dark rhythm for 7 days. During this period, the germination energy (GE), germination rate (GR), germination index (GI), and seeding vigor index (SVI) of the seeds were calculated by the following formulas, according to ISTA [11]:

$$\text{GE } (\%) = \frac{\text{number of germinated seed at the 3 rd days}}{\text{total number of seeds}} \times 100 \quad (2)$$

$$\text{GR } (\%) = \frac{\text{number of germinated seed at the seventh days}}{\text{total number of seeds}} \times 100 \quad (3)$$

$$GI = \sum (Gt / Tt) \quad (4)$$

$$SVI = GI \times GS \quad (5)$$

where Gt is the number of seeds germinated on day t; and Tt is the number of days; and S is the dry weight of a normal seedling.

2.4. Determination of Soluble Sugar Content

The total soluble sugars were extracted according to the method of Adney and Baker [38]. Specifically, after being ground and homogenized with 10 mL of deionized water, aa 0.5 g sample, along with 5 mL of an 80% ethanol (C_2H_6O) solution, were placed in a water bath at 45 °C for 20 min. Following this, the samples were allowed to cool to room temperature. The homogenate was then centrifuged twice at 6000 rpm for 10 min at 15 °C. Next, 2 mL of the supernatant was combined with 2 mL of 3,5-dinitrosalicylic acid reagent, thoroughly mixed, and then boiled in a water bath for 5 min. After boiling, the mixture was cooled to room temperature in a water–ice bath. The supernatant was collected for the determination of soluble sugar content using a UV spectrophotometer at 540 nm (Shanghai Metash Instruments Co., Ltd., Shanghai, China). Distilled water was used as a control, and glucose was employed to create a standard curve.

The determination method of soluble reducing sugar (SRS) content aligns with the reduction method of 3,5-dinitrosalicylic acid reagent [39]. Sugar extraction involved weighing 0.5 g of the sample in a polypropylene tube, followed by adding 5.0 mL of hexane to wash and remove lipids from the sample. The tube was vortexed for 1 min (Vortex mixer, Biomixer VTX 2500 110 model) and centrifuged at 3220× *g* for 10 min (Eppendorf 580 4R model centrifuge). The supernatant was discarded and 5.0 mL of 80.0% ethanol was added to the sediment. Again, the tube was vortexed for 1 min and centrifuged at 3220× *g* for 10 min. The supernatant was filtered and the volume was adjusted to 10.0 mL with distilled water to obtain the extract. To determine the reducing sugar in the sample, 50.0 µL extract, 200.0 µL NaOH 1M, and 200.0 µL 3,5-DNS were used, and then incubated in a 100 °C water bath for 5 min. After cooling to room temperature, the volume was adjusted to 2.0 mL with distilled water (1550.0 µL). By using a spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China), SRS was quantified using a 1.0 mL quartz test tube and a glucose standard curve at 546 nm (0.02–0.12 mg/mL).

2.5. Determination of Antioxidant Enzyme Activities

Superoxide dismutase (SOD) activity was determined following a previous method [40]. A quantity of 0.5 g fresh seed samples was ground in phosphate-buffered saline (PBS, pH 7.8) and centrifuged at 4 °C for 20 min at 10,500× *g*. The supernatant was mixed with 130 mmol L^{-1} methionine and 750 µmol L^{-1} riboflavin. The mixture was then illuminated in a 4000 LX incubator for 20 min, and the optical density (OD) at 560 nm was measured using a spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China).

The catalase (CAT) activity was determined according to Bailly et al.'s method [41]. The enzyme assay consisted of 0.1 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) with 0.4 mL of enzyme extract in a final volume of 3 mL. The absorbance was recorded at 240 nm at 30 s intervals using a spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China). CAT activity was expressed as a 0.1 change in OD per minute.

The peroxidase (POD) activity was assessed according to Scebba et al.'s method [42]. In detail, 0.5 g fresh seed embryos were ground and extracted in liquid nitrogen. Subsequently, 3 mL of phosphate buffered saline (PBS, pH 7.8) was added to the ice bath. The extract was centrifuged at 12,000× *g* for 10 min at 4 °C. Afterward, a reaction was initiated by adding 1.95 mL of 0.3% H_2O_2, 0.95 mL of 0.2% guaiacol, 1 mL of phosphate-buffered saline (PBS, pH 7.0), and 0.1 mL of the supernatant. The absorbance at 470 nm was recorded using a spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China).

The enzyme activities were calculated using the following formulas [43,44]:

$$\text{SOD activity } (\text{Ug}^{-1}\ \text{FW h}^{-1}) = \frac{(A_0 - A_S) \times V_t \times 60}{A_0 \times 0.5 \times FW \times V_s \times t} \quad (6)$$

where A_0 is the absorbance of the control tube under light; A_s is the absorbance of the sample measuring tube; V_t is the total volume of the sample extraction solution (mL); V_s is the amount of crude enzyme solution extracted during measurement (mL); t is the light exposure time of the color reaction (min); FW is the fresh weight of the sample (g).

$$\text{CAT activity } \left(\text{Ug}^{-1}\ \text{FW min}^{-1}\right) = \frac{\Delta A240 \times V_t}{0.1 \times V_s \times t \times FW} \quad (7)$$

where ΔA240 = (As1 + As2 + As3)/3 is the absorbance of the boiled enzyme solution compared to the control tube; As1, As2, and As3 are the absorbance values of the sample measuring tube; V_t is the total volume of enzyme extract (mL); V_s is the volume of enzyme solution taken during measurement (mL); FW is the fresh weight of the sample (g).

$$\text{POD activity } \left(\Delta OD470\ \text{g}^{-1}\ \text{FW min}^{-1}\right) = \frac{\Delta A470 \times V_t}{FW \times V_s \times t} \times n \quad (8)$$

where ΔA470 is the change in absorbance value of A470 within 3 min; V_t is the total volume of enzyme extract (mL); V_s is the volume of enzyme solution taken during measurement (mL); FW is the fresh weight of the sample (g); t is the reaction time; n is the dilution ratio.

2.6. Determination of the Endogenous Hormone Content

The reagent test kits ml147100, ml020295, ml062451, and ml064270, were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China) and used to detect the content of auxin (IAA), cytokinin (CTK), gibberellin (GA), and abscisic acid (ABA) in the fresh seed embryos, following their respective instructions. The OD values of IAA, CTK, GA, and ABA were all measured at a wavelength of 450 nm using a spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China). The measurements were performed in triplicate.

2.7. Statistical Analysis

Statistical analysis was performed using Excel 2016 (Microsoft Office, Washington, DC, USA) and SPSS v.26 statistical package (SPSS, Chicago, IL, USA). The test of normal distribution was conducted using the "nortest" packages in Rstudio software (Version: 2024.04.2+764, https://posit.co/downloads/ accessed on 10 June 2024). Differences in the above testing parameters, as affected by variety, harvest period, and year, and their interactions, were examined using a three-factor model of analysis of variances (ANOVA). When the ANOVA was proven significant for any parameter, a least significant difference (LSD) test was assayed for multiple comparisons at $p \leq 0.05$. Correlation analysis was performed using the "linkET" packages in Rstudio software to investigate the relationships between seed vigor traits, antioxidant enzyme activities, phenotypes, and physiological traits [45]. Data in figures and tables were the average of three replicates.

3. Results

3.1. Phenotypic and Moisture Characteristic Changes in Seeds and Embryos at Different Harvest Stages

As the black layer was visible at the base of the kernel until 35 DAP for JNY6F and 50 DAP for PH4CV, that meant the kernels and embryos had reached physiological maturation (PM) (Figure 1A), and the embryos had also reached the maximal size at those time points, when the structure of the cotyledon, coleoptile, plumule, plumular axis, radicle, and seminal root primordia were all visible, suggesting that the embryo morphology had been fully established (Figure 1B).

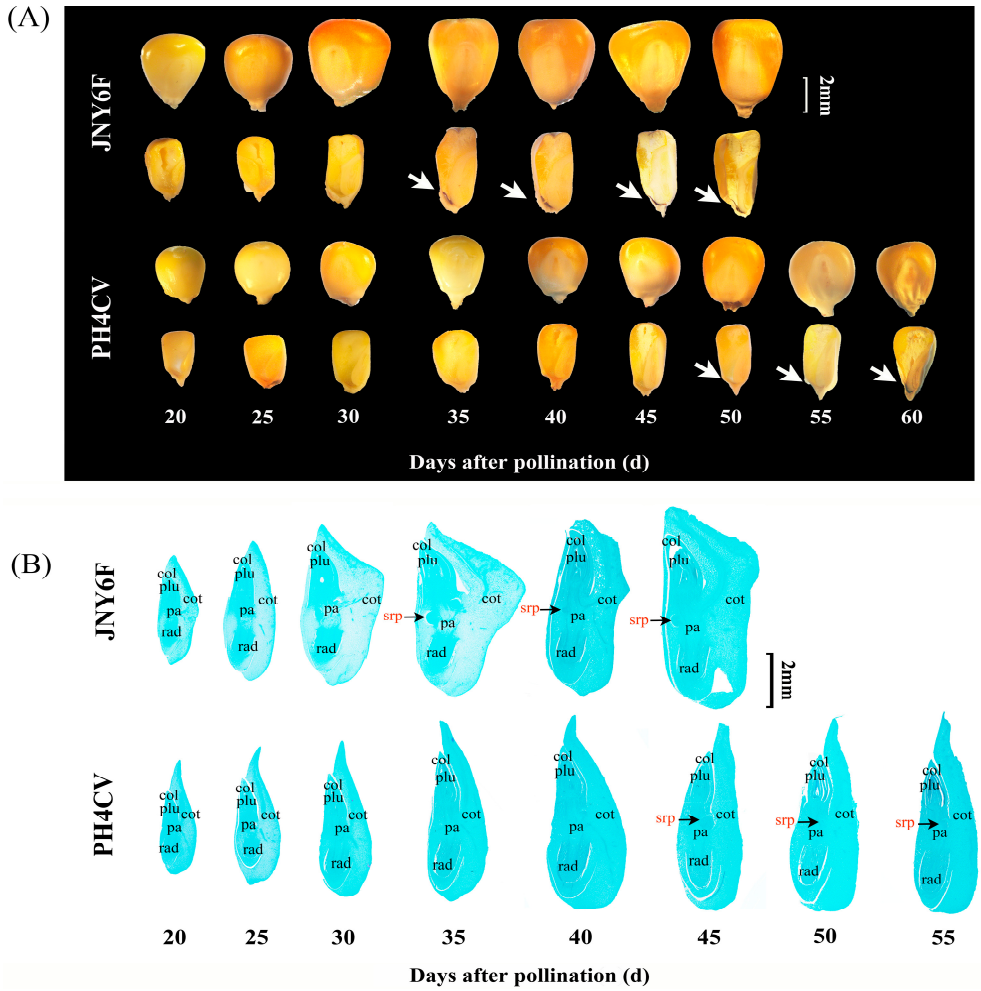

Figure 1. Phenotypic changes of the seeds and embryos of two maize inbred lines at different maturity stages in 2020 and 2021. (**A**) Dynamic changes of seed phenotype of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. The white arrows indicate the black layers of maize seeds. (**B**) Dynamic changes of embryo phenotype of JNY6F and PH4CV from 20 DAP to 50 DAP and 20 DAP to 60 DAP, respectively. cot, cotyledon; col, coleoptile; plu, plumule; pa, plumular axis; rad, radicle; srp, seminal root primordia.

The ANOVA results indicated that moisture content (MC) of seeds and embryos was significantly affected by variety (V)), harvest stage (H), year (Y) and Y × H, but other interactions between the two factors had different effects on the seed fresh weight (Figure 2, Table S1). In two years, the fresh weights of JNY6F and PH4CV seeds both continued to increase from 20 DAP to 35 DAP and 20 DAP to 45 DAP, then slightly decreased, and finally remained stable form 45 DAP to 50 DAP and 55 DAP to 60 DAP, respectively (Figure 2A). Except for JNY6F in 2020, the seed dry matter accumulation (SDMA) continuously increased from 20 DAP to 50 DAP; that of JNY6F in 2021 and PH4CV over the two years first increased from 20 DAP to 35 DAP and 20 DAP to 45 DAP, with an average increase of 157.6% and 241.7%, indicating that these periods were the critical grain-filling stages for JNY6F and PH4CV, respectively. Subsequently, the rates stabilized from 35 DAP to 50 DAP for JNY6F and from 45 DAP to 60 DAP for PH4CV. Conversely, the fresh and dry weights of embryos of JNY6F and PH4CV reached the maximum values at 40 DAP and 50 DAP within two years, and then showed rapid and slow decline patterns, respectively (Figure 2B). The above results indicated that seeds and embryos of maize inbred lines had entered the dehydration and desiccation stage from 20 DAP, and the fresh weight and dry matter accumulation of

seeds and embryos of maize inbred lines might reach the maximum values close to the PM time.

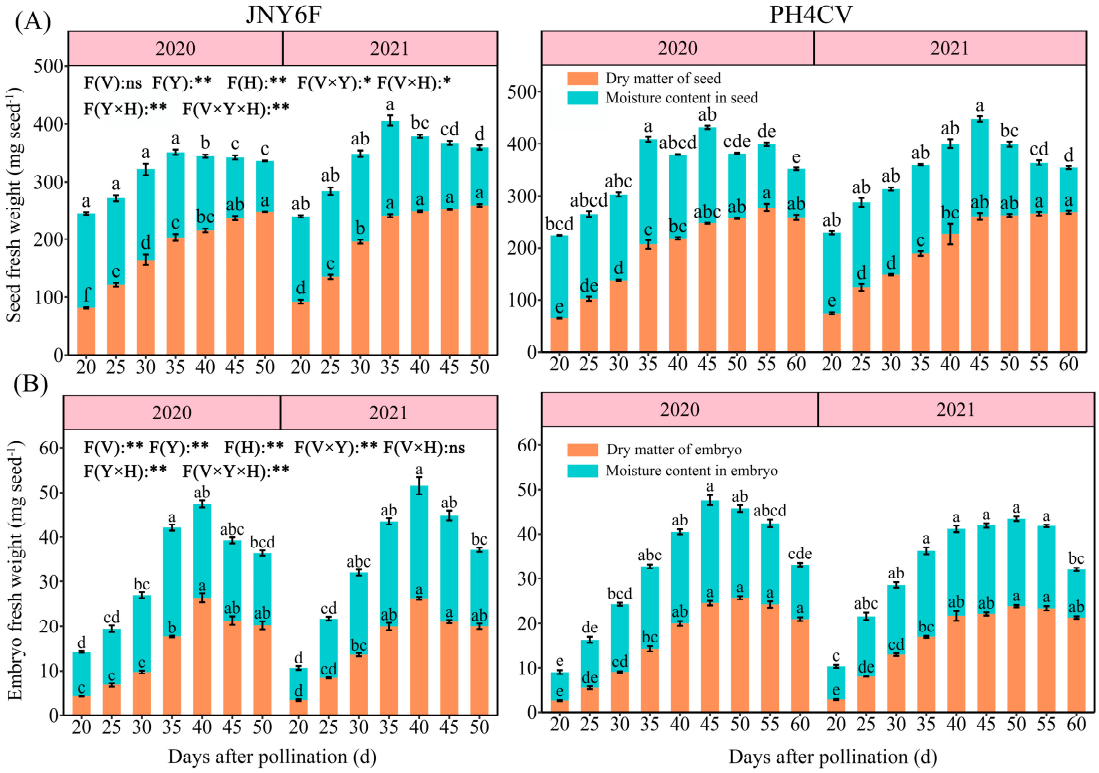

Figure 2. Effects of maturity stage on dry matter accumulation and moisture content of seeds and embryos of two maize inbred lines in 2020 and 2021. (**A**) Dynamic changes of seed dry matter accumulation and moisture content of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**B**) Dynamic changes of embryo dry matter accumulation and moisture content of JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. ns indicates no significant difference, and * and ** indicates significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively. The different lowercase letters indicate significant differences among different harvest stages at the $p < 0.05$ level. The error bar indicates the standard error from three biological replicates.

The variations in the fresh and dry weight of seeds were related to the dynamic changes of seed moisture content in seed (MCIS); that of JNY6F decreased slowly from 20 DAP to 35 DAP, and reduced sharply from 35 DAP to 50 DAP, with an average decrease of 32.9%, while the MCIS of PH4CV remained stable from 20 DAP to 45 DAP, and then both decreased continuously (Figure 2A). Moreover, the moisture content in embryos (MCIE) of JNY6F and PH4CV continued to increase from 20 DAP to 35 DAP and 20 DAP to 45 DAP, with an average increase of 179.7% and 209.4%, and the maximum values were 24.0 mg·embryo^{-1} and 21.5 mg·embryo^{-1}, respectively, and then both declined continuously (Figure 2B). For the moisture content (MC), the seed MC of JNY6F and PH4CV decreased continuously from an average of 64.0% to 27.6%, and 68.9% to 25.9%, and the embryo MC decreased continuously from an average of 68.8% to 46.4%, and 71.5% to 34.0% within two years, respectively (Figure 2). These results demonstrated that the period of rapid dehydration of maize inbred seeds and embryos was approaching PM time.

3.2. Changes of Seed Vigor in Maize Inbred Lines at Different Harvest Stages

The ANOVA results indicated that the four seed vigor indexes, including germination energy (GE), germination rate (GR), germination index (GI), and seedling vigor index (SVI), were all affected by year (Y), harvest time (H), and V × Y × H, but other interactions

between the two factors had different effects on the four seed vigor indexes (Figure 3, Table S1). The standard germination results showed that the four seed vigor indexes of PH4CV were higher than that of JNY6F at the same harvest time within both years, and the change trends of GE, GR, GI, and SVI of specific maize inbred lines were highly similar and complex. For instance, the GE, GR, GI, and SVI of JNY6F all had two peaks at 35 DAP and 45 DAP, respectively, but that of PH4CV only had a peak at 50 DAP within both years, and the occurrence times of the first two peaks were related to PM time (Figure 3). Notably, the maximum values of GE, GR, GI, and SVI occurred at 35 DAP for JNY6F with an average of 82.2%, 100%, 107.6, and 22.5, and 50 DAP for PH4CV with an average of 95.7%, 98.5%, 108.2, and 25.9 within both years, respectively (Figure 3). It is confusing that the GE, GR, GI, and SVI of both maize inbred lines significantly decreased within the following 5 days after the occurrence time of the first peaks, and then significantly increased. Above all, we set the final date (35 DAP and 50 DAP) to reach maximum GE, GR, GI, and SVI as the timely harvest times for JNY6F and PH4CV, respectively, because the seed vigor of those time sfor JNY6F and PH4CV were the highest.

3.3. Physiological Trait Changes in Fresh Seed Embryos at Different Harvest Stages

To elucidate the regulatory mechanisms of changes in the seed vigor of maize inbred lines, which decreases initially and then increases after PM, as the embryo is the nascent form of a plant, we detected the soluble sugar, antioxidant enzyme activity, and hormone levels in fresh seed embryos at different harvest stages.

3.3.1. Soluble Sugar Content

The ANOVA results revealed that the total soluble sugar content (TSSC) and soluble reducing sugar content (SRSC) were both significantly affected by the factors variety (V), year (Y), harvest time (H), and Y × H and V × Y × H (Figure 4, Table S1). In the two years, the TSSC and SRSC in fresh embryos of both maize inbred lines reduced continuously as time went on, and the average values of TSSC of JNY6F and PH4CV decreased about 37.7% and 37.6% from 279.4 mg/g (20 DAP) to 174.1 mg/g (50 DAP) and 331.6 mg/g (20 DAP) to 206.8 mg/g (60 DAP), respectively. The soluble reducing sugars of JNY6F and PH4CV decreased from 20.6 mg/g (20 DAP) to 3.6 mg/g (50 DAP) and 11.7 mg/g (20 DAP) to 4.4 mg/g (60 DAP), respectively, with a decrease of 82.5% and 62.3% (Figure 4).

3.3.2. Antioxidant Enzyme Activity

The ANOVA results revealed that the superoxide dismutase activity (SODA), peroxidase activity (PODA), and catalase activity (CATA) were both significantly affected by the year (Y) factor (Figure 5, Table S1). The antioxidant enzyme activity (SOD activity, SODA; CAT activity, CATA; POD activity, PODA) in fresh embryos of two inbred lines exhibited complex patterns along with an increase in seed maturity within two years (Figure 5). For instance, the SODA of JNY6F displayed a pattern of initial increase with an average increase of 60.1% (20 DAP to 25 DAP), followed by a decrease with an average decrease of 33.0% (30 DAP to 35 DAP), and then a subsequent increase with an average increase of 48.6% (35 DAP to 50 DAP), within both years. On the other hand, PH4CV exhibited a fluctuating or wave-like change pattern. In addition, the PODA of JNY6F continued to increase from 20 DAP to 35 DAP, with average values from 7.9 μg^{-1} min^{-1} to 15.3 μg^{-1} min^{-1}, with an average increase of 94.1% in two years, and then decreased rapidly until 40 DAP and slowly increased after that stage, but that of PH4CV continued to rise from 20 DAP to 60 DAP, and the average values continued to increase from 8.1μg^{-1} min^{-1} to 21.1 μg^{-1} min^{-1}, with an average increase of 160.4%. Moreover, the CATA of JNY6F and PH4CV continued to increase until reaching the peaks at 45 DAP and 40 DAP, and the average value of CATA increased from 79.3 μg^{-1} min^{-1} to 263.5 μg^{-1} min^{-1}, with an average increase of 232.0% and 110.2 μg^{-1} min^{-1} to 252.2 μg^{-1} min^{-1} with average increase 232.0% respectively.

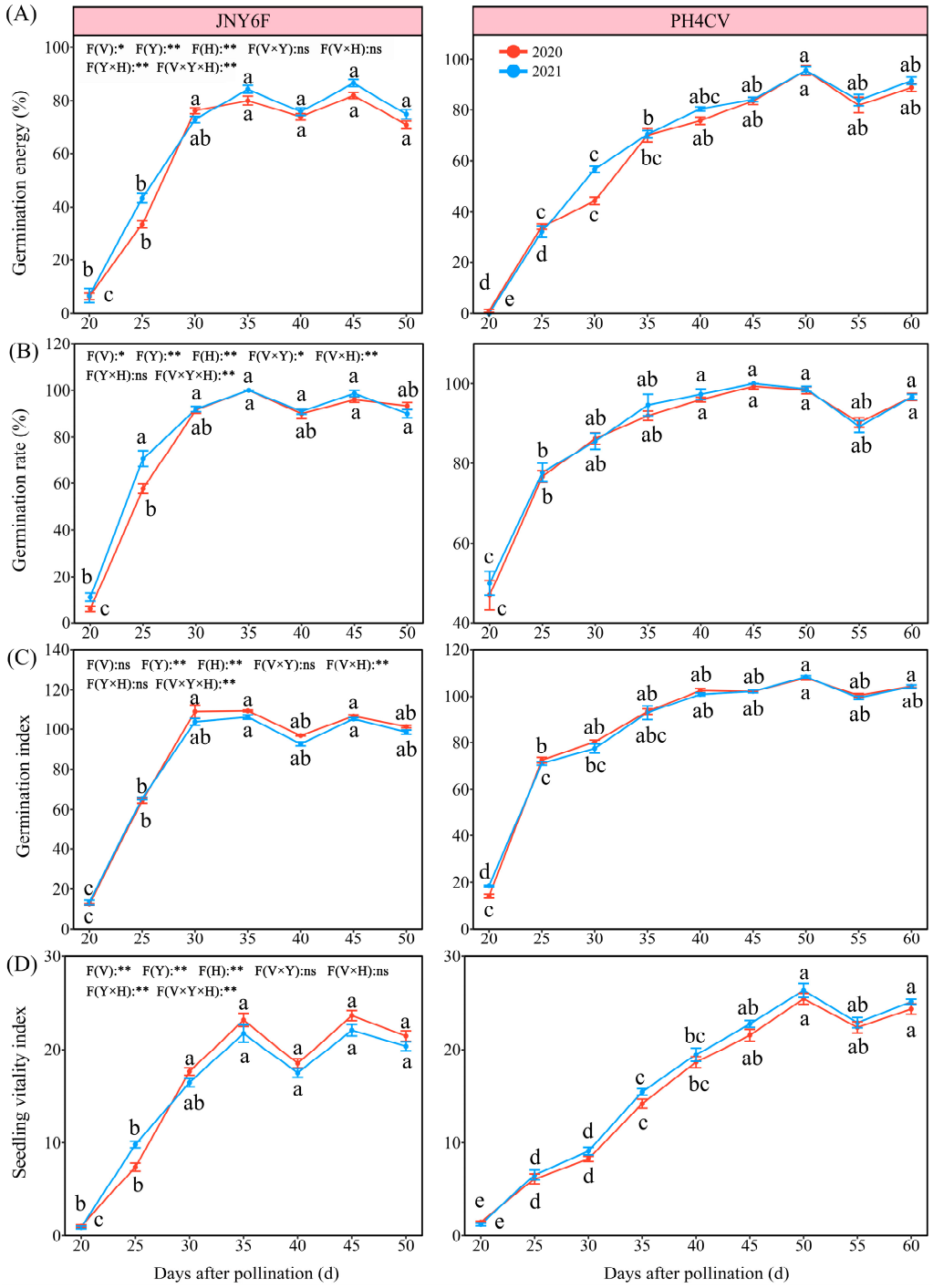

Figure 3. Effects of maturity stage on seed vitality traits of two maize inbred lines in 2020 and 2021. (**A**) Dynamic changes in seed germination energy of maize inbred lines JNY6F and PH4CV, 20 to 50 days and 20 to 60 days after pollination. (**B**) Dynamic changes in seed germination rate of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**C**) Dynamic changes in seed germination index of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**D**) Dynamic changes in seed vitality index of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. ns indicates no significant differences, and * and ** indicate significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively. The different lowercase letters indicate significant differences among different harvest stages at the $p < 0.05$ level. The error bar indicates the standard error from three biological replicates.

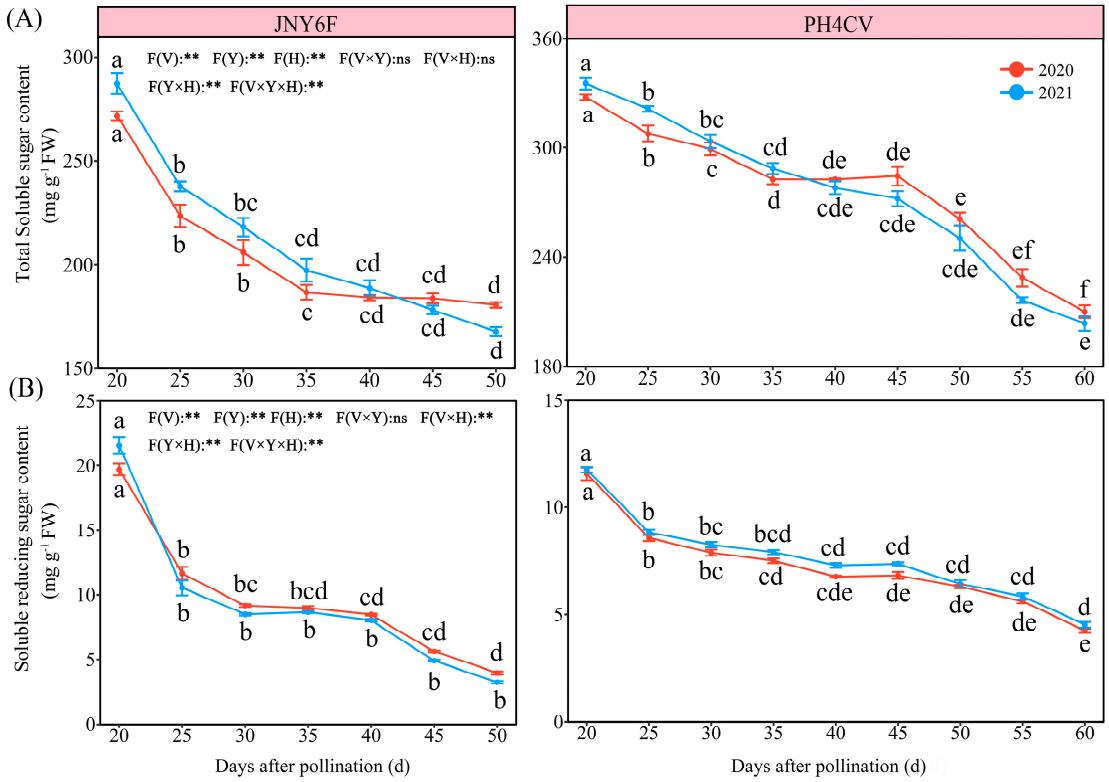

Figure 4. Effects of maturity stage on total soluble sugar and soluble reducing sugar contents in fresh embryos of two maize inbred lines in 2020 and 2021. (**A**) Dynamic changes of total soluble sugar in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**B**) Dynamic changes of soluble reducing sugar in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. ns indicates no significant difference, and ** indicates significant differences at the $p < 0.01$ level. The different lowercase letters indicate significant differences among different harvest stages at the $p < 0.05$ level. The error bar indicates the standard error from the biological replicates.

3.3.3. Hormone Levels

The concentrations of four phytohormones, including auxin (IAA) content (IAAC), cytokinin content (CTKC), gibberellin content (GAC), and abscisic acid content (ABAC), were evaluated in fresh seed embryos of JNY6F and PH4CV. The ANOVA results indicated that the four phytohormone levels were all affected by variety (V), year (Y), and harvest time (H), but the interactions between the two or three factors had different effects on them (Figure 6, Table S1). Over the course of two years, the IAAC of JNY6F and PH4CV exhibited a declining trend, characterized by a gradual decrease in the early stages and a more rapid decline in the later stages. The average IAAC peaked at 20 DAP, with average values of 477.4 nmol g^{-1} and 414.3 nmol g^{-1} for JNY6F and PH4CV, respectively. For CTKC, JNY6F showed an initial increase from 20 DAP to 25 DAP, followed by stabilization, and then a continuous decrease. Conversely, PH4CV decreased initially from 20 DAP to 25 DAP and then exhibited an increase until 30 DAP, reaching its maximum value, and decreased gradually after this stage. The peak CTKC values for JNY6F and PH4CV occurred at 25 DAP and 30 DAP, with mean values of 900.3 ng g^{-1} and 892.3 ng g^{-1}, respectively. The changes in GAC differed between the two maize inbred lines. In addition, the GAC of JNY6F stabilized after increasing from 20 DAP to 25 DAP, and then rapidly decreased after 35 DAP. However, the GAC of PH4CV was stable from 20 DAP to 35 DAP, with mean values of 1546.9 pmol g^{-1}, and then reduced gradually until 60 DAP by about 52.6%. Similarly, the ABAC of JNY6F continued to rise from 20 DAP to 35 DAP, with an average increase

of 12.9% over two years, and then decreased gradually until 50 DAP by about 48.4%. In contrast, the ABAC of PH4CV was stable from 20 DAP to 35 DAP, and reached the peak value of 1244.1 ng g^{-1}, and then decreased until 60 DAP by about 67.1%. These results demonstrated that the GAC and ABAC in fresh embryos of the two maize inbred lines were highly correlated with the change patterns of seed vigor, which reached the peaks close to PM time.

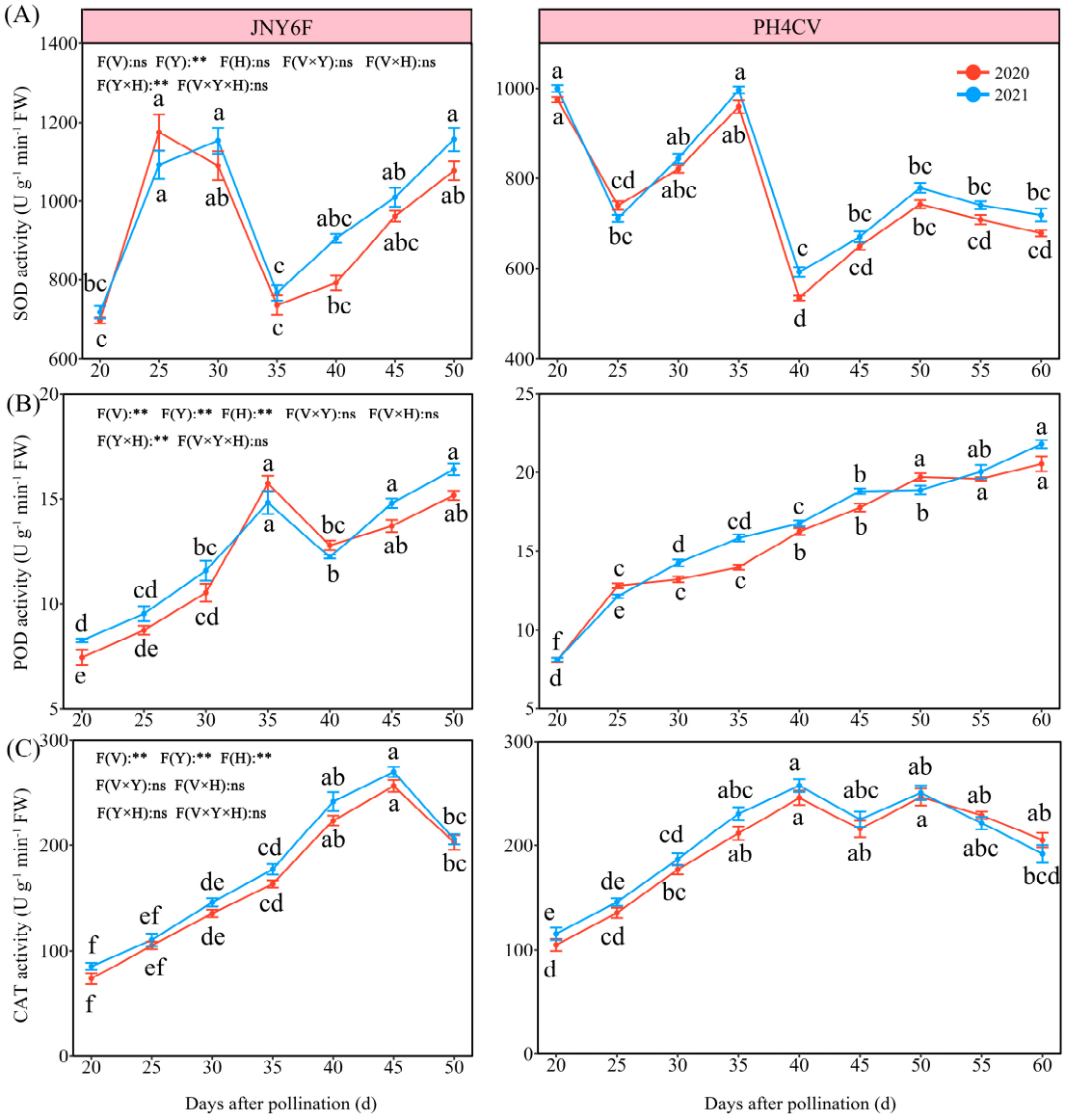

Figure 5. Effects of maturity stage on antioxidant enzyme activities in fresh embryos of two maize inbred lines in 2020 and 2021. (**A**) Dynamic changes of superoxide dismutase activity in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**B**) Dynamic changes of peroxidase activity in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**C**) Dynamic changes of catalase activity in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. ns indicates no significant difference, and ** indicates significant differences at the $p < 0.01$ level. The different lowercase letters indicate significant differences among different harvest stages at the $p < 0.05$ level. The error bar indicates the standard error from three biological replicates.

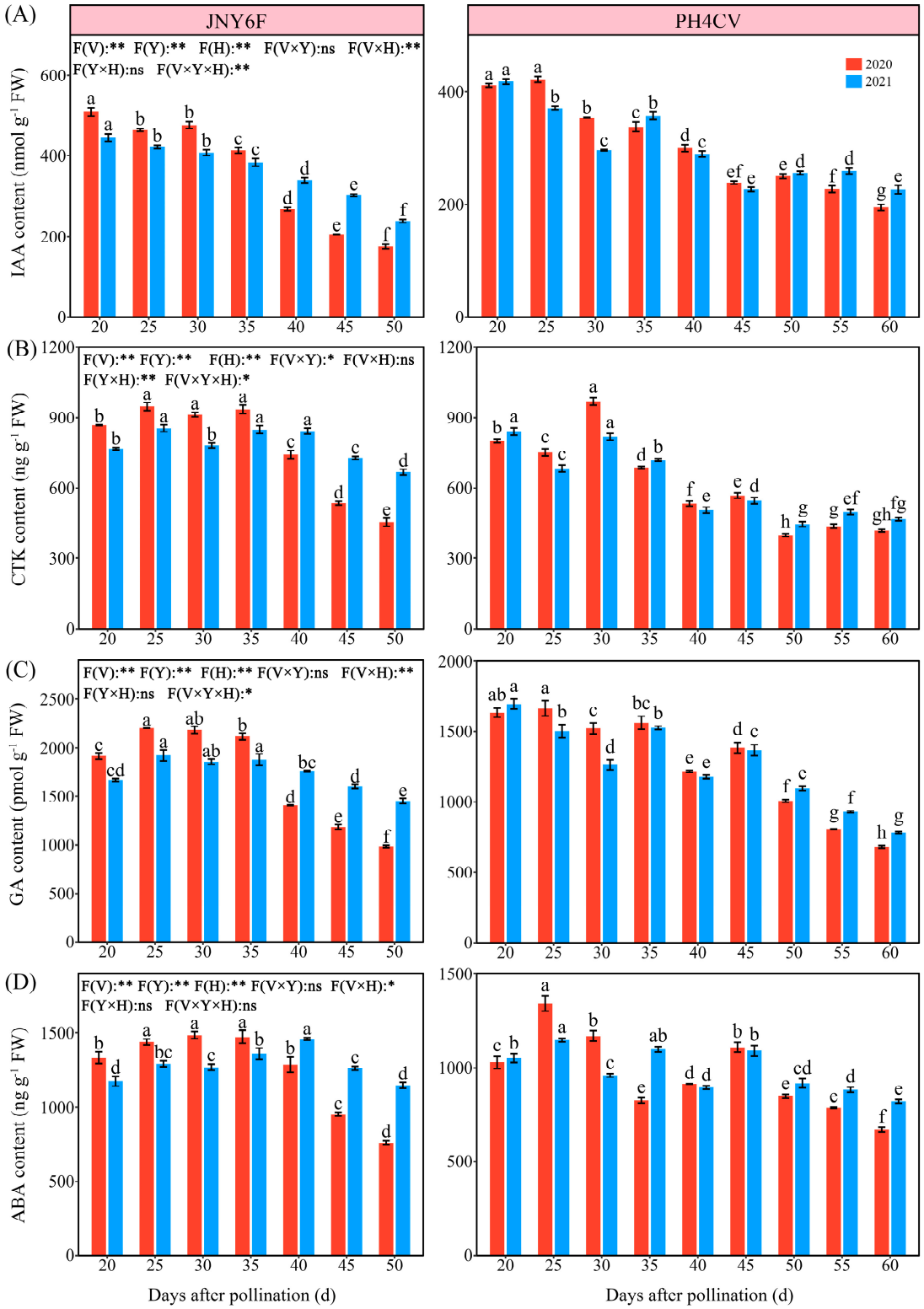

Figure 6. Effects of maturity stage on endogenous hormone content in fresh embryos of two maize inbred lines in 2020 and 2021. (**A**) Dynamic changes of auxin content in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**B**) Dynamic changes of cytokinin content in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively.

(**C**) Dynamic changes of gibberellin content in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. (**D**) Dynamic changes of abscisic acid content in fresh embryos of maize inbred lines JNY6F and PH4CV from 20 days after pollination (DAP) to 50 DAP and 20 DAP to 60 DAP, respectively. ns indicates no significant differences, and * and ** indicate significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively. The different lowercase letters indicate significant differences among different harvest stages at the $p < 0.05$ level. The error bar indicates the standard error from three biological replicates.

3.4. Correlation among the Seed Vigor Indexes and Phenotypic and Physiological Traits

As shown in Figure 7, there were significant positive correlations between GE, GR, GI, SVI and SDW, EDW, EAMC, CATA, PODA, and significant negative correlations between GE, GR, GI, SVI and SMC, EMC, SRSC, TSSC, IAAC, CTKC, GAC, ABAC. Moreover, GR and SVI were significantly negatively correlated with SODA and MCIS, respectively. Furthermore, there were significant positive or negative correlations among most physiological indicators and phenotypic traits. These results indicated that the measured physiological indicators, including soluble sugar, antioxidant enzyme activity, and hormonal levels, were involved in regulating seed and embryo establishment and seed vigor of maize inbred lines with different mechanisms.

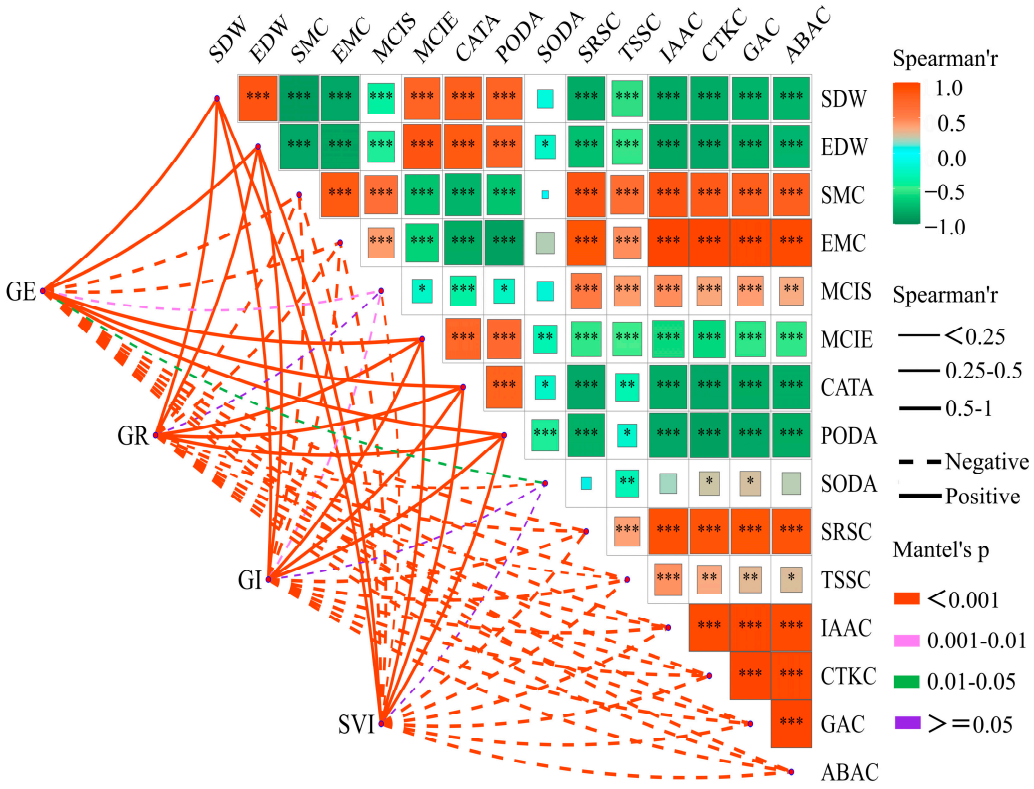

Figure 7. The relationship among seed vigor indexes, antioxidant enzyme activities, and endogenous hormone contents. GE, germination energy; GR, germination rate; GI, germination index; SVI, seeding vitality index; SDW, seed dry weight; EDW, embryo dry weight; SMC, seed moisture content; EMC, embryo moisture content; MCIS, moisture content in seed; MCIE, moisture content in embryo; CATA, catalase (CAT) activity; PODA, peroxidase (POD) activity; SODA, superoxide dismutase (SOD) activity; SRSC, soluble reducing sugar content; TSSC, total soluble sugar content; IAAC, auxin content; CTKC, cytokinin (CTK) content; GAC, gibberellin (GA) content; ABAC, abscisic acid (ABA) content. The color gradient denotes Spearman's correlation coefficient. The line width denotes Spearman's r value. The line color denotes the statistical significance level. *, **, *** indicates significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

4. Discussion

4.1. Timely Harvest Is a Prerequisite for Ensuring the Seed Vigor of Maize Inbred Lines

Timely harvest is crucial for ensuring the seed vigor of hybrid maize, as it directly influences seed quality and subsequent plant performance [29,30,32]. Recent studies have demonstrated that the seed vigor increases as dry matter accumulates during seed development, which reaches the peak value at a stage close to the physiological maturity (PM) time [46]. Hence, if seeds are not harvested in time after the PM time, the seed vigor will decline, affected by seed desiccation damage [47]. Previous studies have shown that harvesting the seeds in time obtained the maximum seed vigor of rice [48] and maize varieties [29,30,32]. In this study, we detected that the highest seed vigor of JNY6F (early maturity) and PH4CV (late maturity) displayed at 35 DAP and 45 DAP, respectively. This is consistent with a recent report, which indicated that the later-maturity maize hybrid varieties reached the ideal harvest time (IHT) later than the early-maturity varieties, as the whole growth period of the later-maturity varieties is longer [32].

Seed moisture content (SMC) is a key indicator that has been investigated in relation to the IHT for maize hybrid seeds. Several studies have indicated that maize seeds exhibit the highest vigor when the SMC is within a certain range, around 35% [32,46,49]. However, in a previous study for maize inbred lines, it was demonstrated that the SMC was simultaneously influenced by time point, growing environment, and genotype–environment interaction [50]. In the present study, we discovered that the SMC of JNY6F and PH4CV at IHT were 41.4% at 35 DAP or 30.0% at 45 DAP (as two seed vigor peaks appeared) and 33.2% at 50 DAP, and the embryo moisture content (EMC) of JNY6F and PH4CV were 56.9% at 35 DAP or 51.6% at 45 DAP and 45.6% at 50 DAP (Figure 2), respectively. These findings suggest that the SMC of maize inbred lines at IHT is significantly different from that of maize hybrids, even among different inbred lines, and the EMC remains at a higher level than the SMC at this time.

The four seed vigor indexes were highly negatively correlated with SMC (Figure 7). Regarding the observed complex variation in seed vigor in this study, showing an initial decrease followed by an increase after PM (Figure 3), we speculate that this could be attributed to the higher SMC during this stage (Figure 2). Natural drying immediately after PM may result in rapid dehydration of the seeds, leading to significant damage affecting the seed vigor. However, if a certain period of time, such as ten days, is allowed to pass, the SMC will relatively decrease. Consequently, during the process of natural drying, the lower SMC would inflict less damage to the embryo.

4.2. The Soluble Sugar Content of Fresh Embryos Is Not the Key Factor for Maintaining Seed Vigor after Drying

Seed germination and seedling growth require a significant amount of energy and nutrients. During seed germination, the stored carbohydrates and other chemical components in the seeds are degraded into small molecules to maintain internal osmotic balance and provide the necessary substances and energy for the seedling growth [51]. Soluble sugars play a role in protecting membrane permeability [52]. For instance, monosaccharides and raffinose have been investigated to be closely related to seed vigor, and the levels of these soluble sugars significantly decrease as seed vigor declines [53]. A recent study demonstrated that the soluble sugar content of maize seed continued to decrease from 8 DAP [54]. However, a previous study indicated that the soluble sugar composition, rather than the total content, was crucial for membrane stabilization, and the presence of raffinose at specific levels could protect seeds during high-temperature drying [55]. In this study, we found that the total soluble sugars and reducing sugars were all negatively correlated with four seed vigor indexes (Figure 7), as the total soluble sugar and reducing sugar content of fresh embryos both decreased continuously during seed development (Figure 4), which was consistent with a recent study [54]. Thus, we hypothesize that the specific soluble sugar compositions synthesized during seed development may really participate in

maintaining internal osmotic balance, improving seed germination and seedling growth, but this hypothesis need further investigation.

4.3. Antioxidant Enzymes in Developing Embryos Play Important Roles in Ensuring Seed Vigor after Drying

Seeds encounter oxidative stress during development, maturation, and germination [56]. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which are generated during seed aging and deterioration processes, play crucial roles in scavenging reactive oxygen species (ROS) [57]. Reactive oxygen species (ROS) are continuously produced throughout the entire seed development process, from embryonic development to germination and storage, as demonstrated in references [58,59]. In the current study, peroxidase activity (PODA) in the fresh embryos of JNY6F exhibited an initial increase from 20 to 35 days after pollination (DAP), followed by a decrease. In contrast, in PH4CV, PODA continuously rose from 20 DAP to 60 DAP. Additionally, the catalase activity (CATA) in the fresh embryos of JNY6F and PH4CV displayed a gradually increasing trend, peaking at 45 DAP and 40 DAP, respectively (Figure 5). In addition, the correlation analysis results showed that the PODA and CATA were highly positively related to four seed vigor indexes, but SODA was not significantly related with them. Therefore, we present a hypothesis that POD and CAT accumulated in development embryos may serve to counteract the damage caused by free oxygen radicals produced during seed dehydration, and help protect the seeds from oxidative damage to maintain the seeds with high vigor.

4.4. Pythormones Participate in Establishing Embryo Morphogenesis to Improve Seed Vigor

Phytohormones are crucial regulatory factors in seed development and germination [11]. Numerous reviews have demonstrated the pivotal roles of pyhtohormones synthesized during seed development in regulating seed germination, and provide insights into the internal hormone-signaling networks that control this process. For instance, auxin influences embryo development by regulating cell division, elongation, and differentiation, and also plays an important role in seed dormancy release [60,61]. Gibberellins (GAs) are essential for seed germination by promoting embryo development and regulating various developmental processes during seedling establishment and growth [62,63]. Abscisic acid (ABA) inhibits seed germination by promoting seed dormancy [18,64]. Cytokinins (CTKs) influence embryo growth and differentiation by promoting cell division and shoot development. Additionally, there are interactions among these pyhtohormones, as CTKs interact with ABA and GAs to facilitate seed germination [22–26], and auxin positively regulates ABA biosynthesis and negatively regulates GA biosynthesis to inhibit seed germination [27,28]. In addition, several studies have demonstrated that hormone expression and function precede the emergence of phenotypic traits [65]. In this study, a significant negative correlation was observed between the levels of four pythormones (IAA, CTK, GA, ABA) in immature embryos and the four indexes of seed vigor (Figure 7). It was noted that the levels of these four pythormones in immature embryos of two maize inbred lines remained relatively stable from 20 DAP to 30 or 35 DAP before gradually decreasing (Figure 6). In light of the phenotypic analysis and changes in seed vigor indexes, the four seed vigor indexes of maize inbred line JNY6F all exhibited peak values by 35 DAP (Figure 3). Hence, it is reliable to believe that the stable hormonal expression promoting early embryo morphogenesis for JNY6F. However, for the maize inbred line PH4CV, it was challenging to understand the relationship between hormone content and the fact that the four seed vigor indexes reached their maximum values at 50 DAP (Figure 3). Studies have emphasized the critical importance of precise timing and levels of hormone expression for optimal functional performance, as excessive or insufficient levels can lead to contrasting effects [66]. For PH4CV, the three seed vigor indexes (GE, GR, GI) from 20 to 30 or 35 days was the rapid growth phase, although the levels of the four hormones in its immature embryos continued to decrease after 30 or 35 days, it is likely that their levels did not

hinder their role in promoting embryonic morphogenesis. This complexity underscores the intricate mechanisms involved in seed vigor formation, which may differ among different maize genotypes, particularly in terms of plant hormones. However, further in-depth research is needed to validate these speculations.

5. Conclusions

Establishing embryo morphology is essential for maintaining high seed vigor for maize inbred lines. Two different maturity maize genotypes exhibited distinct seed vigor change patterns, but both showed an initial increase followed by a decrease, peaking near physiological maturity (PM). Throughout embryo development, soluble reducing sugars and total soluble sugars decreased continuously, and were negatively correlated with seed vigor indexes. In fact, there may not be significant interrelationships among them. The catalase (CAT) and peroxidase (POD) activities increased until reaching maximum values close to PM. These enzyme activities were significantly correlated with seed vigor indexes, suggesting that seed vigor may be influenced by the accumulation of antioxidants through varying mechanisms. In addition, the timing and levels of expression of the four hormones may play a key role in promoting embryonic morphogenesis and seed vigor formation, and the seed vigor mechanisms between different genotypes may vary due to dynamic changes in hormone levels. Moving forward, exploring the interplay of these factors and their regulation mechanisms could provide valuable insights into enhancing seed vigor for sustainable agriculture practices.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14081770/s1; Table S1: The analysis of variance results of the seed vigor indexes and phenotypic and physiological traits of two maize inbred lines.

Author Contributions: Conceptualization, W.F. and W.W.; Methodology, W.F., W.W., Z.S. and J.X.; Software, W.F., H.W. and Y.B.; Formal analysis, W.F., H.W., W.W., Z.S., P.Q. and Y.G.; Investigation, W.F., P.Q. and Y.G.; Data curation, H.W. and W.X.; Writing—original draft, W.F., H.W., Y.B. and W.X.; Writing—review & editing, W.F., H.W., Y.B. and H.M.; Visualization, Y.B.,W.X., Z.S., P.Q. and Y.G.; Supervision, J.X. and M.S.; Project administration, W.F.; Funding acquisition, J.X., M.S. and H.M.; Validation, H.W. and Y.B.; Resources, W.F., M.S. and H.M. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are greatly thankful to the Research Program Sponsored by the Ministerial and Provincial Co-Innovation Centre for Endemic Crops Production with High-quality and Efficiency in Loess Plateau (SBGJXTZX), the Natural Science Foundation of Shanxi (20210302123372), the Biological Breeding Engineering projects of Shanxi Agricultural University (YZGC090), and the Graduate Practice Innovation Project of Shanxi Province (2023KY329) for financial support.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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