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Development of a paper-based microfluidic biosensor for the colorimetric detection of malate dehydrogenase in maize seeds

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ABSTRACT: Seed deterioration is a major constraint on germination efficiency and overall crop productivity, while conventional quality assessment methods are often laborious and time-consuming. In this study, a microfluidic paper-based analytical device (μPAD) was developed for rapid and cost-effective evaluation of maize seed quality via malate dehydrogenase (MDH) activity. The colorimetric assay is based on NAD^+ (nicotinamide adenine dinucleotide) reduction and subsequent redox reaction with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)/PMS (phenazine methosulfate), producing a purple signal proportional to enzyme activity. Optimal conditions included a 3 mm flow aperture, 10 mg/ml MTT, 20 min incubation, and 30 μl sample volume. The biosensor exhibited a concentration-dependent response and a clear decline in signal with seed aging, correlating well with germination rate. Statistical analysis confirmed significant differences ($p < 0.015$). The proposed μPAD demonstrated high analytical sensitivity for detecting MDH in low-concentration seed extracts, achieving a limit of detection (LOD) and limit of quantification (LOQ) of 0.23 mg/ml and 0.70 mg/ml, respectively. This μPAD provides a portable and reliable platform for real-time seed vigor assessment, offering a practical alternative to conventional laboratory methods.

KEYWORDS: seed quality, microfluidic paper-based analytic device (μPAD), malate dehydrogenase, seed deterioration, colorimetric detection

INTRODUCTION

Seed deterioration remains one of the most significant challenges in global agriculture, as it directly impacts germination uniformity, seedling vigor, and ultimate crop yields [1]. This physiological decline is a complex, irreversible process characterized by the accumulation of metabolic byproducts, membrane disruption, and a progressive loss of enzymatic efficiency [2]. In the context of maize (*Zea mays*), which serves as a primary global staple and industrial resource, ensuring the distribution of high-vigor seeds is essential for maintaining food security and economic stability [3]. Currently, the International Seed Testing Association (ISTA) germination assay is the industry standard for quality assessment [4]. However, these biological assays are inherently time-consuming [5, 6], often requiring a seven-day incubation period before definitive results can be obtained, which is incompatible with the fast-paced demands of modern seed processing [7]. While the Tetrazolium (TZ) test offers a more rapid alternative for viability assessment, it relies on hazardous chemicals and necessitates subjective manual interpretation by highly trained personnel, often leading to variability in results [8]. Furthermore, advanced technologies such as X-ray [9], computed tomography (CT) [10], and hyperspectral imaging [11] provide non-destructive analysis but are hindered by high instrumentation costs and a lack of accessibility for routine industrial applications.

Consequently, there is a critical need for a determination platform that bridges the gap between analytical accuracy, safety, and cost-effectiveness. MDH (EC 1.1.1.37) has been identified as a highly reliable biochemical biomarker for monitoring the process of seed deterioration [12]. MDH is an essential enzyme in the tricarboxylic acid (TCA) cycle that catalyzes the interconversion of malate and oxaloacetate. Its activity is strongly correlated with the percentage of germination and respiratory capacity of the maize embryo [13]. Because MDH is fundamental to mitochondrial respiration and energy production during the earliest stages of germination, its activity level serves as a sensitive indicator of physiological age and overall seed viability [14].

This study reports the development of a novel paper-based analytical device designed for the rapid biochemical assessment of maize seed quality through the quantification of MDH activity. The proposed biosensor utilizes a 3D microfluidic architecture to fa-

cilitate controlled reagent interaction and uniform signal development [15]. Enzymatic biosensors were utilized as precise analytical tools to monitor enzymatic activity [16]. These devices integrated a redox reagent transducer to convert biochemical interactions into measurable signals. The signal output correlated with the concentrations of substrates or cofactors, therefore offering a distinct optical representation of the target analyte concentration [17]. This was achieved by integrating a colorimetric enzymatic redox system based on NAD^+, malic acid, and the chromogenic tetrazolium salt (MTT). The sensor subsequently translated MDH activity into a quantifiable change in color intensity. Unlike traditional spectrophotometric methods that require equipment and large sample volumes, this paper-based approach offered a resource-efficient, non-hazardous, and reliable alternative. This platform demonstrated significant potential for implementation in routine quality control, supporting strategic agricultural initiatives by providing a practical tool to ensure the integrity of the maize seed industry.

MATERIALS AND METHODS

Reagents

The biosensor employed a colorimetric enzyme reaction. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenazine methosulfate (PMS), beta-nicotinamide adenine dinucleotide (NAD^+), and L-malic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Buffer components, including tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), potassium phosphate monobasic (KH_2PO_4), and potassium phosphate dibasic (K_2HPO_4), were also obtained from Sigma-Aldrich.

Plant materials and controlled deterioration treatment

Commercial maize seeds obtained from a local store were subjected to an artificial aging protocol to simulate natural seed deterioration under accelerated conditions. Seeds were incubated in a controlled environmental chamber at 40 °C with a constant relative humidity of 90%. To determine the aging of seed viability, samples were incubated at specified intervals (0, 1, 2, 3, 4, 5, 6, 7, 8, and 10 days). Following treatment, a standard germination test was performed according to ISTA guidelines [18]. Three replicates of 100 seeds were placed on moistened filter paper and maintained at 30 °C with a 12-h light/12-h dark photoperiod. The germination percentage was recorded after seven days with successful germination defined as a radicle protrusion of at least 1 mm. All experiments were conducted using the same batch of seeds to ensure experimental consistency and direct comparability, including biosensor and standard enzymatic methods.

Design and fabrication of the microfluidic paper-based analytical device (μPAD)

The biosensor was engineered as a 3D-structured μPAD to facilitate uniform reagent distribution and clear colorimetric result. The device architecture comprised three main functional layers assembled on a laminating plastic substrate. The detection layer consisted of 6 mm-diameter paper discs (Western blotting filter paper, Thermo Scientific, USA), while the bottom fluidic layer utilized Whatman No. 1 filter paper precision-cut via a CO_2 laser. These layers were integrated using a double-sided adhesive 3M pad with CO_2 laser cuts to regulate the vertical flow of the sample (Fig. 1a). All components were assembled on laminate plastic by 2-sided adhesive tape. The standard reagents were applied to the bottom pad as shown in Fig. 1b, including MTT (10 mg/ml, 0.2 μl), PMS (3 mg/ml, 0.1 μl), NAD^+ (3 mg/ml, 2.0 μl), and malic acid (25 mg/ml, 0.2 μl).

Preparation of maize seed extract and biosensor detection

Maize seed samples were homogenized for 1 min using a mechanical grinder (DeLonghi, 150 W). To extract the target enzymes, 10 mg of the resulting seed powder was suspended in 1 ml of 100 mM Tris-HCl buffer (pH 8.5), vortexed for 1 min, and centrifuged (8,000×g, 10 min, 4 °C). For the biosensor assay, 30 μl of the supernatant was applied to the sample zone, followed by an incubation period of 20 min at ambient temperature. The assay was based on the activity of MDH, which catalyzes the oxidation of malic acid to oxaloacetate with the concomitant reduction of NAD^+ to NADH, producing a visible color change from colorless to purple. The intensity of this color is proportional to the MDH enzymatic activity. The resulting colorimetric intensity, indicative of enzymatic activity, was captured using a high-resolution flatbed scanner (Canon CanoScan LiDE 400, Canon Inc., Japan) and quantified via digital image grayscale analysis using ImageJ software. To quantify MDH activity and reduce background signal, the differential mean grayscale intensity was calculated. The mean grayscale value of the control system without malic acid was subtracted from the mean grayscale value of the complete reaction mixture. The differential mean grayscale intensity (Δ Intensity) was calculated as follows: Δ Intensity = grayscale value (with malic acid) − grayscale value (without malic acid).

To calculate the inter-day relative standard deviation (%RSD), three distinct biosensor devices were used over three separate days to measure the MDH activity of control sample seeds. For intra-day repeatability, four replicate measurements were performed to detect MDH activity of control sample seeds within a single analytical session.

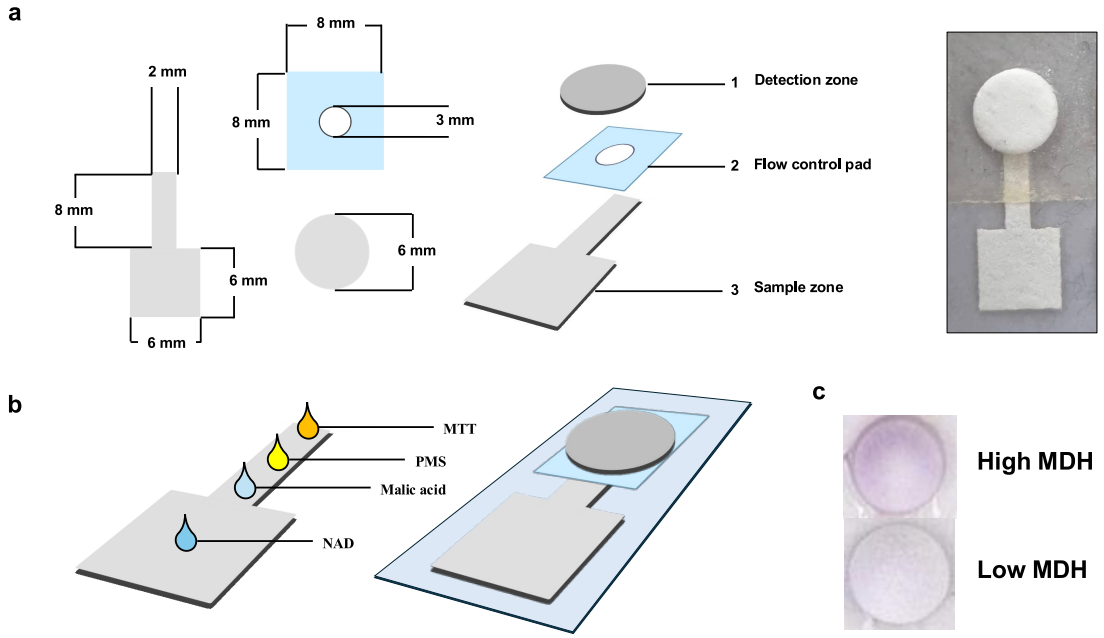

Fig. 1 Schematic overview of the μPAD: (a) components of the μPAD; (b) fabrication process of the μPAD; (c) colorimetric comparison of high and low MDH activity on the biosensor.

Optimization of μPAD architecture and reagent composition

The μPAD was optimized by evaluating physical and chemical parameters to maximize sensitivity. To determine the ideal flow rate, the diameter of the aperture in the middle flow-control pad was varied (1, 2, 3, and 5 mm) using CO_2 laser ablation. The chemical matrix was optimized by testing six reagent formulations, ranging from single components to a complete system (MTT, PMS, NAD^+, and malic acid). To determine the optimal reagent composition for the paper-based biosensor, six distinct formulations were evaluated: (1) MTT, (2) MTT + PMS, (3) MTT + PMS + NAD^+, (4) MTT + PMS + malic acid, (5) MTT + PMS + NAD^+ + malic acid, and (6) PMS + NAD^+ + malic acid. Each reagent mixture was incorporated into the reaction zone of the biosensor and tested using maize seed extracts prepared according to the established extraction protocol. The analytical performance of each configuration was assessed in quadruplicate to identify the synergistic effects of the mediators (MTT and PMS), the co-factor (NAD), and the substrate (malic acid) on signal development. This comparative analysis allowed for the determination of the most effective chemical composition for maximizing catalytic efficiency and detection sensitivity. Furthermore, the concentration of the chromogenic substrate, MTT, was evaluated to identify the concentration providing the highest signal-to-noise ratio. To optimize the enzymatic reaction rate and electron transfer efficiency of the paper-based biosensor, the effect of MTT concentration was evaluated across a range of 0.5 to 10 mg/ml. For each concentration, a fixed volume of the MTT solution was applied to the reaction zone and allowed to dry, after which the biosensor response was measured in quadruplicate to ensure statistical reproducibility.

Characterization of enzymatic reaction parameters for biosensors

The influence of environmental and operational variables on the MDH reaction was systematically investigated. The optimal pH was determined by testing a range of 7.4–8.5, while the effect of temperature was assessed at 25, 30, and 35 °C. To define the assay kinetics, incubation times between 10 and 35 min were evaluated. Additionally, the biochemical response was characterized by varying the substrate (malic acid) concentration and the initial seed extract concentration (0.5–100 mg/ml). The volume of the sample applied to the biosensor was also standardized by comparing the performance of 10, 30, and 50 μl aliquots.

Study of correlation between seed aging and MDH detection

To evaluate the testing ability of the μPAD, the relationship between physiological seed age and the biosensor signal was systematically analyzed. Extracts from the aging gradient (0–10 days) were applied to the optimized sensors in quadruplicate. The resulting colorimetric shift caused by the enzymatic reduction of MTT to formazan was captured using a high-resolution flatbed scanner. The digital images were processed via ImageJ software to determine the mean grayscale

value of the detection zones.

Validation using commercial microplate assay kit

For analytical validation, a colorimetric microplate assay was performed using a FlexStation® 3 Multi-Mode Microplate Reader to monitor reaction kinetics. A comparative analysis was conducted using the CheKine™ Micro NAD-MDH Activity Assay Kit (Abbkine, China). Briefly, 0.1 g of seed sample was homogenized in 1 ml of extraction buffer and centrifuged (8,000×g, 10 min, 4 °C), and the supernatant was analyzed. MDH activity was determined by measuring the change in absorbance at 340 nm.

RESULTS

Colorimetric biosensor response and reagent composition optimization

The colorimetric reaction of the biosensor is based on the enzymatic activity of MDH, which catalyzes the conversion of malate to oxaloacetate coupled with the reduction of NAD^+ to NADH. The generated NADH subsequently participates in a redox reaction with the chromogenic reagent, leading to a visible color change. In this system, the color transition was observed from colorless to purple, corresponding to the level of MDH activity (Fig. 2c).

Initially, the biosensor produced a strong colorimetric response, indicating substantial MDH activity on day 0. By day 10, there was a noticeable decrease in signal intensity (Fig. 1c), indicating a significant decrease in enzyme activity (Fig. 2a). This negative trend is consistent with the germination data given in Fig. S1, which shows a temporal decline in seed vigor. As a result, the significant association between biosensor output and physiological performance supports MDH activity as a reliable biochemical diagnostic of seed quality.

The effect of reagent composition on biosensor performance was presented in Fig. 2b. The selected reagent mixture was optimized to achieve maximum sensitivity and signal stability. In this work, the chosen composition (MTT, PMS, NAD^+, and malic acid) provided an optimal balance between enzyme cofactor availability (NAD^+), substrate concentration, and chromogenic reagent efficiency, ensuring sufficient NADH generation and effective color development. Deviations from this composition resulted in either weak signal intensity or unstable color formation, likely due to insufficient reaction kinetics or reagent imbalance. Therefore, this optimized formulation was selected for subsequent experiments, as it provides the most reliable and reproducible response.

Evaluation of the analytical precision and reproducibility of the MDH biosensor

The precision of the MDH activity assay was evaluated by determining both the intra-day and inter-day relative standard deviations (%RSD). For the intra-day assessment, four replicate measurements were performed on four distinct devices within a single analytical session, yielding an average %RSD of 7.18%. To evaluate inter-day precision, measurements were conducted across three different devices over a period of three consecutive days. The analysis demonstrated high consistency with an inter-day %RSD of 7.03%. These values fall within the acceptable threshold for microfluidic and spectrophotometric biosensing applications, indicating that the assay maintains robust reproducibility despite potential fluctuations in ambient conditions or device-to-device variability.

Environmental influences on sensor performance

To better understand environmental effects, optimization of pH and temperature was performed using both the presence and absence of malic acid (w/o malic acid), where the latter represents the background signal in the absence of MDH activity.

The biosensor exhibited a strong pH dependence with maximum performance at pH 8.5 (Fig. 3a). At this pH, the difference between the signal (with malic acid) and background (w/o malic acid) was maximized. This behavior can be attributed to the intrinsic properties of MDH, which shows higher catalytic activity under slightly alkaline conditions. At lower or higher pH values, enzyme conformation and active site integrity was affected, leading to reduced catalytic efficiency and smaller signal-to-background differences. Additionally, the background signal remained relatively stable across pH conditions, highlighting that pH primarily influences enzymatic activity rather than non-specific reactions.

Temperature optimization further demonstrated its critical role in biosensor performance (Fig. 3b). The maximum performance was found to be 25 °C, where the highest signal intensity and best signal-to-background separation were achieved. At elevated temperatures (30–35 °C), the signal decreased. This reduction is likely due to a combination of factors, including decreased enzyme stability, accelerated solvent evaporation in the paper matrix, and disruption of reaction kinetics. These effects collectively reduce the efficiency of NADH generation and subsequent color development.

Optimization of reaction conditions and device parameters

The optimization of the reaction system confirmed that all four components (MTT, PMS, NAD^+, and malic acid) were essential for effective colorimetric signal generation. Control experiments lacking any individual component resulted in significantly reduced color development, verifying the requirement of a complete redox chain for signal production. In this study, PMS and NAD^+ were maintained in excess to ensure that the reaction was not limited by electron mediators or

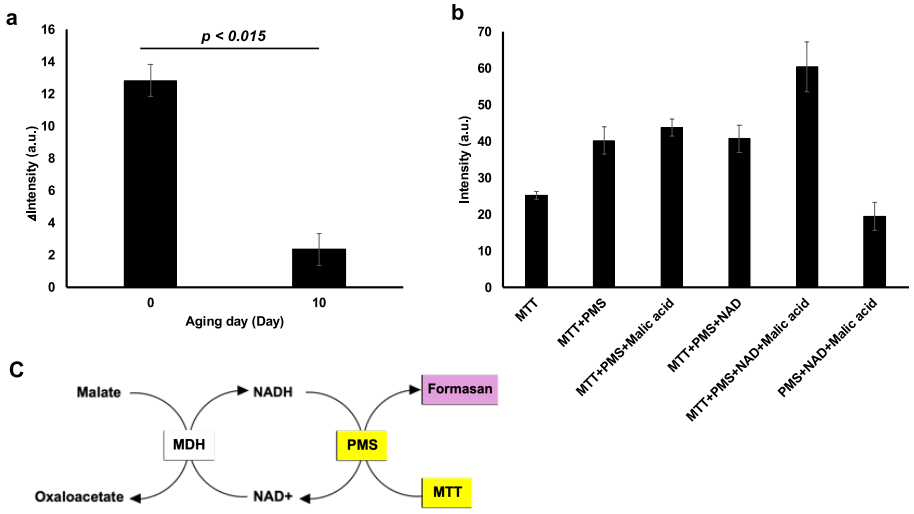

Fig. 2 Evaluation of biosensor performance: (a) comparison of seed aging effects on Day 0 and Day 10; (b) reagent effect on biosensor performance; (c) reaction scheme of MDH/MTT/PMS system.

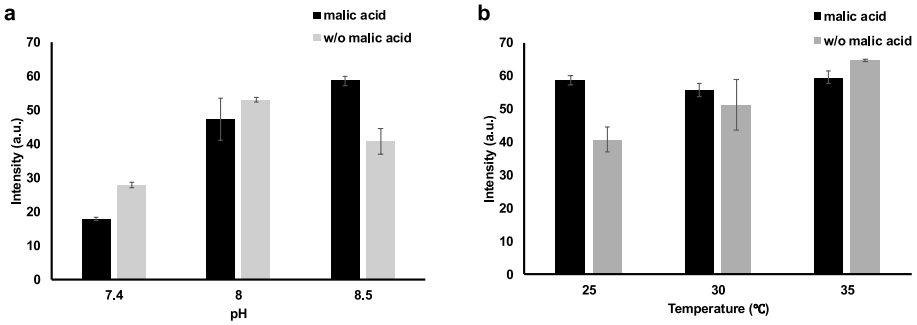

Fig. 3 Evaluation of experimental conditions affecting biosensor performance: (a) pH; (b) temperature.

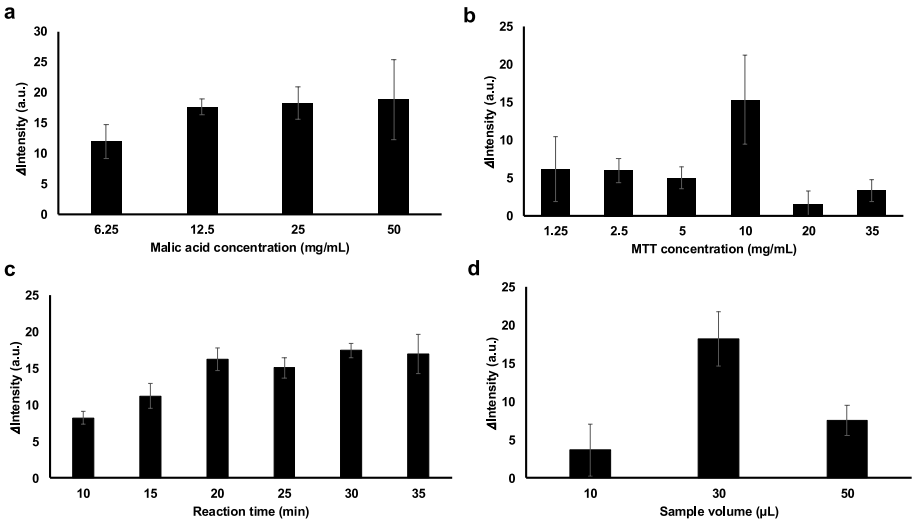

Fig. 4 Optimization of key reaction parameters affecting biosensor performance: (a) malic acid concentration; (b) MTT concentration; (c) incubation time; (d) sample volume.

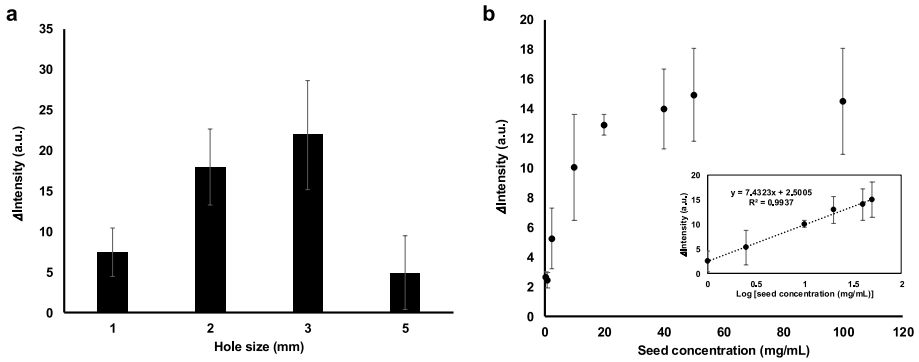

Fig. 5 Investigation of design and sample parameters influencing biosensor performance: (a) hole size of the flow control pad; (b) sample concentration.

cofactors, allowing the system to specifically evaluate the effects of malic acid and MTT.

The biosensor response showed a clear dependence on malic acid concentration (Fig. 4a). The optimal condition was determined to be 25 mg/ml (0.2 µl), which provided the highest and most consistent signal. This result confirms that the system can effectively detect variations in MDH activity across a wide dynamic range.

MTT concentration was identified as a critical parameter influencing signal intensity (Fig. 4b). Increasing MTT concentration led to a proportional enhancement of the colorimetric response up to 10 mg/ml, beyond which no improvement was observed. This optimal concentration likely reflects efficient electron transfer from NADH via PMS to MTT, resulting in stable and intense color formation with minimal background interference.

Although PMS and NAD^+ were not systematically varied, their roles are essential in facilitating electron transfer and cofactor regeneration within the redox cycle. Their use in excess ensures that the observed signal is primarily governed by substrate (malic acid) availability and chromogenic response (MTT), rather than by limitations in reaction intermediates.

Incubation time was identified as a critical parameter influencing assay performance. The colorimetric signal exhibited a time-dependent increase, gradually intensifying until reaching a stable plateau at approximately 20 min (Fig. 4c). Beyond this duration, no significant enhancement in signal intensity was observed, suggesting that the enzymatic reaction had reached completion. Accordingly, an incubation time of 20 min was selected as the optimal condition, providing an appropriate balance between analytical sensitivity and assay efficiency.

Sample volume was a critical parameter influencing fluid distribution, interaction efficiency within the detection zone, and overall signal consistency. Optimization studies revealed that a volume of 30 µl was sufficient to achieve complete and uniform coverage of the detection area. Volumes below this threshold resulted in insufficient wetting, while higher volumes caused overflow, leading to signal distortion and reduced measurement reliability (Fig. 4d).

Precise control of fluid dynamics within paper-based analytical devices is essential for achieving consistent and reliable biosensor performance. In this work, flow-control aperture size was found to influence signal intensity. An optimal aperture diameter of 3 mm produced the highest response (Fig. 5a). Smaller diameters likely restricted fluid flow and limited reagent transport, while larger diameters reduced residence time, leading to incomplete reaction development. Similarly, the sample volume was optimized to balance sufficient reagent interaction and controlled flow within the paper matrix.

The effect of seed extract concentration (0.5–100 mg/ml) on biosensor performance showed a clear concentration-dependent increase in signal intensity, corresponding to enhanced enzymatic activity. A sharp increase at low concentrations (0.5–10 mg/ml) indicated enzyme-limited conditions, followed by a gradual rise at intermediate levels, suggesting partial system saturation. At higher concentrations, the signal plateaued, likely due to limitations in reagent diffusion or saturation of the paper matrix. Accordingly, 10 mg/ml was selected as the optimal concentration to maximize sensitivity while avoiding saturation effects (Fig. 5b). Overall, these results demonstrate that both reaction composition and device design play critical roles in achieving optimal biosensor performance.

The calibration performance of the µPAD biosensor was evaluated by analyzing the relationship between seed extract concentration and the resulting change in color intensity, which demonstrated a non-linear saturation curve typical of enzymatic biosensing platforms. By applying a logarithmic transformation to the seed concentration, a highly linear response was achieved ($y = 7.4323x + 2.5005$), yielding a strong coefficient of determination ($R^2 = 0.9937$). This robust linearity within the calibrated range confirms the

reliability of the sensing mechanism for quantification. Furthermore, the analytical sensitivity was substantiated by calculated LOD and LOQ of 0.23 mg/ml and 0.70 mg/ml, respectively (Fig. 5b). These values demonstrate the capability of the developed biosensor to achieve precise detection of MDH activity in a low concentration of sample.

Relationship between biosensor response and germination rate

The biosensor was applied to monitor maize seeds subjected to artificial aging over a period of 0–10 days. A clear and consistent decrease in colorimetric intensity was observed with increasing aging duration (Fig. 6a). The signal intensity declined from 12.83 on Day 0 to 2.35 on Day 10, reflecting a substantial reduction in enzymatic activity. This trend closely matched the decline in germination rate observed under the same conditions. Seeds with high germination potential on Day 0 (90.75%) exhibited the strongest biosensor signal, while severely aged seeds at later time points (e.g., Day 10, germination <20%) showed reduced colorimetric intensity. Notably, both the biosensor response and germination rate displayed a non-linear decrease, with a gradual reduction during the early aging stages followed by a sharp decline after Day 3–5. The relationship between biosensor signal and germination performance indicated a strong correlation between MDH activity and seed viability. The decrease in colorimetric intensity reflects the loss of metabolic activity associated with seed deterioration. These results demonstrate that the developed biosensor can reliably track changes in seed vigor and provide a rapid alternative to conventional germination testing.

Correlation between biosensor response and commercial MDH assay

To validate the performance of the developed biosensor, its response was compared to that of a commercial MDH assay kit. The MDH activity measured by the commercial kit showed a sharp decline with increasing aging time, decreasing from 4508.75 unit/g (Day 0) to undetectable enzyme activity on Day 7, indicating loss of enzymatic activity (Fig. 6b). A similar decreasing trend was observed in the biosensor response, where colorimetric intensity progressively declined with seed aging. During the first few days after aging (Days 0–1), a high level of MDH activity was demonstrated by strong biosensor signals. A low level of MDH activity during the latter part of the incubation period (Days 6–7) produced either no color development or only a very faint color. Although small fluctuations in MDH activity were noted during the intermediate time points (Days 3–5), the overall trends from the two methods were consistent.

The observed temporal decline in analyte activity revealed distinct kinetic profiles between the two platforms. During the initial phase (Days 0–2), the MDH assay exhibited a steep reduction of approximately 90% in activity, whereas the μPAD displayed a more gradual signal attenuation, suggesting a disparity in sensitivity and potential non-linearity at high analyte concentrations. In the intermediate phase (Days 4–6), the MDH assay approached baseline levels, whereas the μPAD maintained detectable signal changes, indicating a lower limit of detection or altered response kinetics. On the other hand, the MDH assay reached zero activity by Day 7, and the μPAD signal plateaued near 2.3–2.5 a.u., a phenomenon attributable to background substrate noise rather than residual analyte activity.

The linear regression analyses showed that both platforms have strong linearity within their respective analytical ranges. The μPAD presented an R^2 of 0.993 across a 0–4 day interval and a slope of -1.7983, which was comparable to the commercial kit ($R^2 = 0.9753$ over a 1–4 day interval) but with a significantly steeper negative slope of -144.44, reflecting a much broader range of absolute values in enzyme activity.

Both methods demonstrated a nonlinear declining pattern of activity over time with an initial rapid loss of MDH activity following the first period of aging. The close agreement of results supports the conclusion that the biosensor accurately reflects changes in enzymatic activity as determined by the standard commercial assay utilized.

DISCUSSION

Artificial aging effectively lowered seed viability, evidenced by a decline in germination rates. These outcomes are consistent with the known roles of oxidative damage and membrane instability in the loss of seed vigor during accelerated aging protocols [19]. Therefore, the applied aging protocol successfully simulates natural seed deterioration and provides a reliable model for evaluating biosensor performance.

The enzymatic assay MDH activity proved to be a sensitive indicator of seed vigor. The observed decrease in color intensity with aging reflects mitochondrial dysfunction, a well-established hallmark of seed deterioration [20]. MDH plays a central role in cellular respiration, and its decline indicates reduced metabolic activity. Compared to traditional TZ testing, which relies on subjective interpretation and manual handling, the developed system offers a more objective and rapid alternative [18].

The optimization of μPAD geometry played a critical role in improving analytical accuracy. The design of a 3 mm hole size minimized the coffee-ring effect and ensured uniform color distribution, which is essential for precise image-based quantification. In a previous study, the flow of the detection zone was also controlled using wax-heating-laser-cutting to enhance color uniformity without any chemical modifications [21]. This phenomenon has been widely

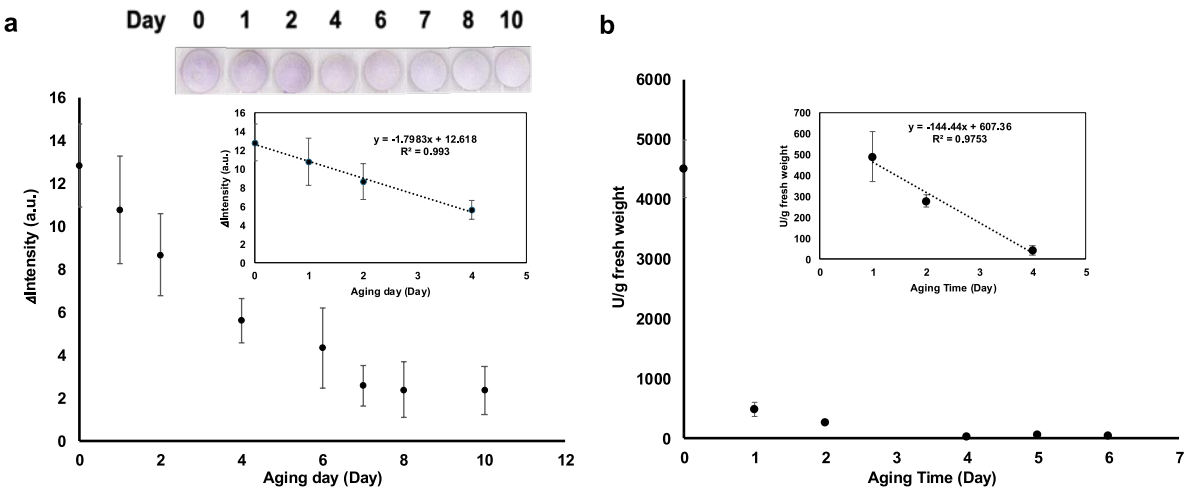

Fig. 6 Correlation between malate dehydrogenase testing methods and seed aging: (a) relationship between color intensity of biosensor and seed aging duration; (b) malate dehydrogenase activity measured by a commercial test kit.

reported in paper-based microfluidic systems, where uneven evaporation leads to solute migration toward the edges [22]. Improving fluid dynamics within the paper matrix significantly enhances reproducibility and signal reliability.

The non-enzymatic reduction of MTT by pyridine nucleotides (NADH and NADPH), mediated by the electron carrier PMS, is a well-recognized source of background interference in dehydrogenase-based assays. In addition, endogenous reductants such as succinate and glutathione can contribute to similar non-specific reduction pathways [23]. In this study, each reagent was deposited separately within the biosensor architecture to minimize unwanted reactions. This spatial separation prevents premature interactions between MTT, PMS, and endogenous reductants, thereby reducing non-specific electron transfer prior to assay initiation and improving the accuracy of the measured signal.

Optimization of reagent composition and operational parameters significantly enhanced biosensor performance. The requirement for a complete redox system (MTT, PMS, NAD^+, and malate) confirms the specificity of the enzymatic reaction. Similar coupled redox systems have been extensively used in colorimetric biosensing due to their high sensitivity and signal amplification capability [24]. The identified optimal conditions, including MTT concentration, incubation time, and sample volume, ensure reproducibility and maximize analytical sensitivity.

Environmental factors such as pH and temperature were found to strongly influence enzymatic activity. The maximum performance at pH 8.5 is consistent with the known alkaline preference of MDH [25]. Likewise, the decline in activity at elevated temperatures (30–35 °C) may be attributed to enzyme instability and increased evaporation rates in paper-based platforms, which has been reported in previous μPAD studies [26]. Consequently, a maximum operating temperature of 25 °C was identified. To ensure analytical reliability, environmental temperature control is required when utilizing this biosensor to prevent thermal-induced fluctuations in performance.

The concentration-dependent response of the biosensor reveals its capacity to quantify enzyme activity over a wide dynamic range. However, the observed signal saturation at high concentrations [27] indicates limitations in reagent diffusion within the paper matrix, emphasizing the need for controlling sample loading to avoid nonlinear responses. In our study, sample volume highly impacts colorimetric detection in paper-based microfluidic systems for a reliable colorimetric assay [28]. The linear detection range of the biosensor was evaluated across seed concentrations of 1–50 mg/ml. Although the linear response extends to 50 mg/ml, a concentration of 10 mg/ml was selected for subsequent assays. This choice optimizes sensitivity while avoiding the signal plateau observed beyond 20 mg/ml, thereby ensuring accurate and reproducible quantification.

The long-term storage stability of paper-based biosensors remains a critical factor for their practical application, particularly since tetrazolium salts can be highly sensitive to environmental factors. The previous report stated that the MTT paper-based biosensor for the detection of glutamate in wine and instant soup showed stability over 6 weeks under storage conditions at −18 °C [29] and the nitro blue tetrazolium (NBT)-based glucose biosensor also showed stability over 8 weeks under storage conditions at −20 °C [30]. However, a paper-based biosensor for the detection of glucose-6-phosphate dehydrogenase,

in which the spontaneous MTT/PMS reaction occurred within 7 days at room temperature, suggested that the biosensor should be freshly prepared before use [31]. To maintain the performance of an MTT-based biosensor, it should be stored under low-temperature conditions.

The correlation between biosensor output and seed aging duration confirms its practical applicability for rapid seed quality assessment. This relationship indicates that MDH activity can serve as a reliable biochemical marker for seed vigor. This study demonstrated that the μPAD was efficient for tracking enzymatic degradation trends, offering a broader dynamic range compared to commercial kits, which typically possess a more limited linear detection range. This highlights a clear trade-off between the portability of paper-based platforms and the comprehensive linear sensitivity of traditional laboratory tests. Consequently, the μPAD is well-suited for rapid field monitoring to validate seed quality, whereas commercial techniques remain the standard for precise enzymatic assessment in the laboratory.

The proposed μPAD was compared with existing standard and analytical methods for seed vigor assessment (Table S1). While traditional approaches such as standard germination testing and spectrophotometry provide established metrics, they are constrained by lengthy assay durations or substantial instrumentation requirements. In contrast, the μPAD platform offers a superior balance of high portability, rapid assay times (20 min), and minimal operational costs, leveraging visual or mobile-based detection that reduces the need for complex laboratories.

In this work, we designed the internal architecture of the sensor to improve signal uniformity while also controlling the flow sequencing and reagent deposition zones to maximize analytical performance and reduce the possibility of spontaneous MTT/PMS redox system reactions. This particularly structural design allows for high-fidelity, real-time seed quality measurement in field situations. Overall, the developed biosensor provides a cost-effective, rapid, and reliable method for assessing seed vigor. Its capacity to provide rapid quantitative data, along with minimal sample preparation and reagent use, makes it a promising alternative to conventional laboratory-based techniques.

CONCLUSION

This study demonstrates the successful development of μPAD as a rapid and cost-effective platform for the evaluation of maize seed quality via MDH activity, which is a biochemical indicator of seed viability. The proposed system enables quantitative assessment by converting enzymatic activity into a measurable colorimetric response, thereby facilitating the clear discrimination between seeds of varying physiological quality. The observed progressive decline in signal intensity with increasing seed aging highlights the capability of the device to effectively monitor seed deterioration. Moreover, the strong agreement between the μPAD response and conventional enzymatic assays confirms its analytical reliability and accuracy. This platform demonstrated operational simplicity, rapid analytical response, and minimal resource requirements, thereby highlighting its potential as a robust and scalable alternative to conventional methodologies for seed quality assessment in agricultural systems.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2026.043.

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Appendix A. Supplementary data

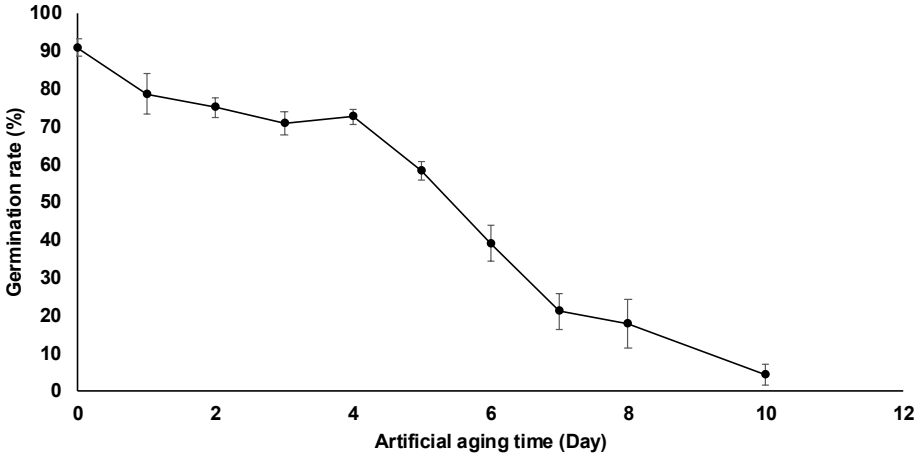

Fig. S1 Germination rate of artificially aged maize seeds. All data are the mean ± standard deviation of three replicates.

Table S1 Comparison of μPAD and standard methods for seed quality testing.

Methodology	Analytical target	Assay duration	Portability	Instrument	Cost	References
Standard germination	Morphological growth	7–14 days	Stationary	Growth chamber	Moderate	[18]
Spectrophotometry	Enzyme kinetics	4–24 h	Low	Spectrophotometer	High	[32, 33]
Tetrazolium test	Dehydrogenase activity	24–48 h	Moderate	Manual microscopy	Low	[34, 35]
μPAD	MDH activity	20 min	High	Visual/Mobile scanner	Low	This work