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Manja Naik E

ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India

Vishwanath Rohidas Yalamalle

vishwanath_yal@yahoo.com

ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India https://orcid.org/0000-0002-5534-0358

Research Article

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Early Post-Imbibition CO_2 Release as a Predictor of Seed Viability: Evidence from a Novel Colorimetric Assay

Manja Naik E[1], Rajeev Kumar[2], Deepak Singh[3], Gyan Prakash Mishra[1], D Vijay[1], Chandu Singh[4], Ganapati Mukri[4], Vijayakumar H P[1], Jitendra[1], Gograj Singh Jat [5]; Bhoopal Singh Tomar[5], Vishwanath Rohidas Yalamalle [1*]

Author for correspondence: Vishwanath Rohidas Yalamalle: vishwantah_yal@yahoo.com

[1]Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India

[2]Division of Agricultural Engineering, ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India

[3]Division of Sample Surveys, ICAR-Indian Agricultural Statistics Research Institute New Delhi, 110012, India

[4]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India

[5]Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India

Abstract

Background Respiration is one of the earliest physiological processes initiated in imbibed seeds, however, its quantitative relationship with seed viability and vigour remains insufficiently explored. The present study aimed to develop a rapid, respiration-based colorimetric assay for assessing seed viability and vigour within a few hours, compared to several days required by conventional germination test.

Results Seed lots with higher germination showed greater CO_2 evolution. Species-specific variation in the CO_2 evolution was studied, which ranged from 487 ppm $seed^{-1}$ in broccoli to 1878 ppm $seed^{-1}$ in green gram. Pre-soaked seeds were placed in airtight tubes containing bromothymol blue (BTB) indicator solution and incubated for 2.5–5 h. CO_2 released by respiring viable seed forms carbonic acid leading to change in the colour of BTB from blue to green or yellow, whereas non-viable seeds leave the solution unchanged (blue). The assay was validated across seven crops: maize, wheat, soybean, green gram, broccoli, pumpkin, and cucumber. Optical density (OD_{450}) values of BTB solution positively correlated with total viability estimated by the standard germination test and vigour level (with r^2 ranging from 0.78 to 1.00).

Conclusion Our findings demonstrate that the seed respiration based colorimetric assay can serve as a rapid, low-cost, and scalable approach for early assessment of seed viability and vigour across wide range of crop species, with potential applications in both seed testing laboratories and research settings.

Keywords Bromothymol blue. colorimetric assay. rapid test. Respiration. seed viability. seed vigour.

Introduction

Seed is the fundamental input for agriculture, and its quality critically determines the success of crop production through its influence on germination capacity, seedling vigour, and field establishment [1,2]. However, seed quality, naturally declines over time, leading to reduced germination, weaker seedlings, and lower yields [3,4]. Maintaining high viability is vital for gene bank conservation, ecological restoration, and the seed industry. Viability varies among species and even within species across locations, habitats, and years [5]. It is influenced by several factors, including natural aging, storage conditions, insect or pest damage, and physical

damage. For farmers, high seed viability ensures good crop establishment, while for seed companies, it is essential for maintaining product quality and commercial success [6].

Seed deterioration or aging is a cumulative and irreversible process that causes cellular damage, leading to a progressive decline in seed vigour and eventual loss of viability [7]. Reactive oxygen species (ROS) like hydroxyl radicals, hydrogen peroxide, superoxide, and singlet oxygen are highly reactive molecules capable of damaging cellular components like membranes, nucleic acids, proteins, carbohydrates, and lipids [9]. The extent of ROS production and accumulation depends on the seed's metabolic and physiological state [10,11]. Excessive ROS induces lipid peroxidation, leading to membrane lipid degradation, loss of membrane integrity, and electrolyte leakage [12]. Ultrastructural damages commonly observed during seed aging include mitochondrial swelling, impaired ribosome dissociation, shortening of the endoplasmic reticulum, and unstacking of Golgi bodies, which collectively contribute to deterioration in seed quality [13,14].

Mitochondria, the primary sites of cellular respiration, play a crucial role in energy generation. ROS-induced damage to mitochondrial membranes disrupts respiration, reduces energy production, and can trigger programmed cell death [15]. In seeds, respiration begins immediately after imbibition and continues until radicle protrusion, making it indispensable for germination [16, 17]. Oxidative respiration, initiated within minutes to hours of water uptake, is essential for mobilizing stored reserves and sustaining metabolic activity [18,19].

The rates of O_2 consumption and CO_2 evolution reflect seed metabolic activity [20] and are strongly correlated with seed vigour and quality [21]. Aged or deteriorated seeds typically exhibit reduced respiration, largely due to ROS-mediated mitochondrial dysfunction, which limits reserve utilization and impairs germination potential [22]. Thus, real-time monitoring of respiratory gases provides valuable insights into the physiological and biochemical basis of

seed vigour [23, 24]. Seed respiration can be quantified using gas sensors and modern seed respiration analysers (SRA), enabling precise measurement of respiration at the individual seed level [25, 26]. The Pettenkofer method, which measures CO_2 release as an indicator of seed respiration, has proven effective for vigour assessment. It enabled reliable classification of soybean seed lots when combined with standard tests [27], CO_2 also showed strong correlations with germination and vigour in okra [28]. Similar results in West Indian gherkin [29] and watermelon [30] confirm CO_2 quantification as a rapid, non-invasive, and robust tool for evaluating seed quality across species.

Seed viability and vigour is evaluated using methods such as the standard germination test, tetrazolium assay, electrical conductivity, X-ray radiography, and embryo excision [31]. Among these, the standard germination test is most widely used, but it requires up to 14 days to complete in field crops, delaying critical decisions for seed companies. It also requires dedicated infrastructure and skilled personnel, which may not be accessible to small-scale stakeholders [32]. The tetrazolium test offers quicker results [31] but relies heavily on technical expertise for interpretation. Overall, conventional methods remain constrained by being destructive, labour-intensive, time-consuming, non-automated, and subject to investigator bias, limiting their efficiency [33].

During the seed respiration the evolved CO_2 can be captured using an acid-base indicator solution, such as bromothymol blue (BTB). BTB is a pH-sensitive indicator widely used as an acid-base indicator [34]. It is a sulfonephthalein compound ($C_{27}H_{28}Br_2O_5S$) with a pKa of around 7.1. The indicator exhibits distinct colour changes depending on its protonation state: in acidic conditions (protonated form), it appears yellow, while in basic conditions (deprotonated form), it turns blue, with a green transition zone between pH 6.0 and 7.6 (Fig. 1).

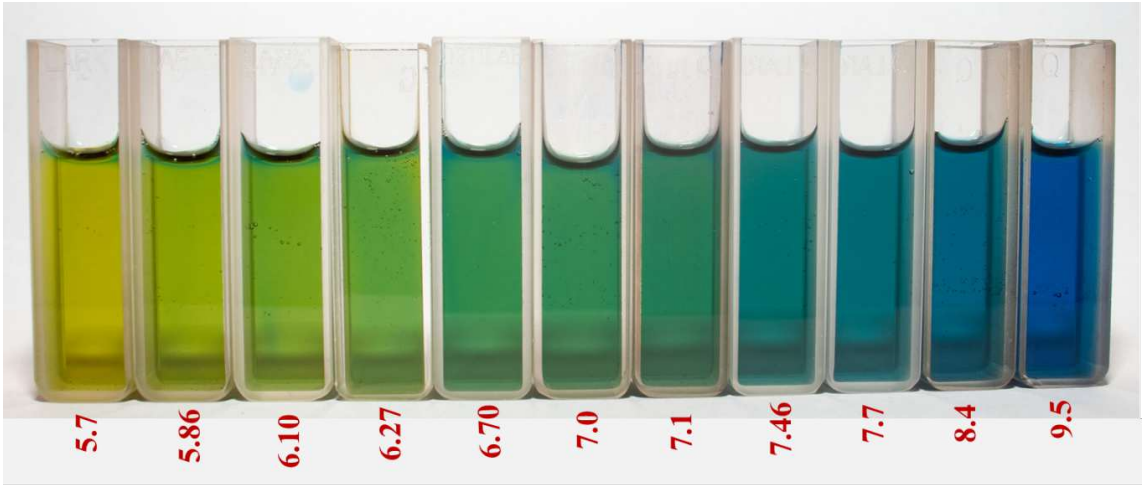


Fig. 1 Colour-change pattern of bromothymol blue across pH: the indicator shifts from yellow in acidic (pH < 6.0) through green around neutral (pH ≈ 7.0) to blue in alkaline (pH > 7.6) solutions.

The CO_2 produced during respiration reacts with the water to form carbonic acid. Carbonic acid (H_2CO_3) is a weak acid formed when CO_2 dissolves in water. The reaction between CO_2 and water produces carbonic acid in a reversible process:

$$CO_2+H_2O \rightleftharpoons H_2CO_3 CO_2 + H_2O \rightleftharpoons H_2CO_3 CO_2+H_2O \rightleftharpoons H_2CO_3$$

Once carbonic acid is formed, it can dissociate into bicarbonate (HCO_3^-) and hydrogen ions (H^+), these ions contribute to the acidity of a solution [34], which in turn leads to the change in the colour of the acid-base indicator.

Bromothymol blue has been widely used as a colorimetric indicator in various biological and environmental applications. It has been utilized as a colorimetric indicator for monitoring the freshness of vegetables during transportation, where CO_2 evolved through respiration induces a visible colour change in BTB [35]. Similarly, Chen *et al*. (2018) [36] reported the use of BTB as an effective freshness indicator in packaged bell peppers, in which increased CO_2 accumulation during storage led to a colour shift. BTB has been used to measure soil respiration [37] and in microbiological assays for detecting bacterial growth through CO_2-induced colour variations [38]. Building on these principles and considering that CO_2 evolution has been

recognized as a reliable indicator of seed vigour in previous studies, the present study aims to develop a rapid BTB-based colorimetric test for assessment of seed viability and vigour.

Materials and methods

Seed materials

Freshly harvested and naturally aged (for 1–3 years) seed lots of maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), soybean (*Glycine max* L.), green gram (*Vigna mungo* L.), broccoli (*Brassica oleracea* var. *italica* L.), pumpkin (*Cucurbita pepo* L.), and cucumber (*Cucumis sativus* L.) were obtained from the Division of Genetics and the Division of Vegetable Science, ICAR–Indian Agricultural Research Institute (IARI), New Delhi.

Measurement of CO_2 evolution

To understand how different crop species release CO_2 during seed imbibition, seeds of maize (n = 3), wheat (n = 5), soybean (n = 3), green gram (n = 3), broccoli (n = 10), pumpkin (n = 3), and cucumber (n = 3) were imbibed with 300 µL of distilled water at 25 ± 1 °C. CO_2 release was recorded for 4 hours using an infrared CO_2 sensor (Model MH-Z19B, Zhengzhou Winsen Electronics Technology Co., Ltd., People's Republic of China) using the Arduino Nano microcontroller (Fig. 2 and Arduino Code S1) with three replications per crop.

To examine how seed lots within a crop having different germination evolve CO_2, measurements were recorded for maize (three varieties) and soybean (four varieties). For both crops, CO_2 release was monitored at 30-minute intervals over 240 minutes in maize and 210 minutes in soybean. The CO_2 evolution was expressed as the average concentration in parts per million per seed (ppm $seed^{-1}$).

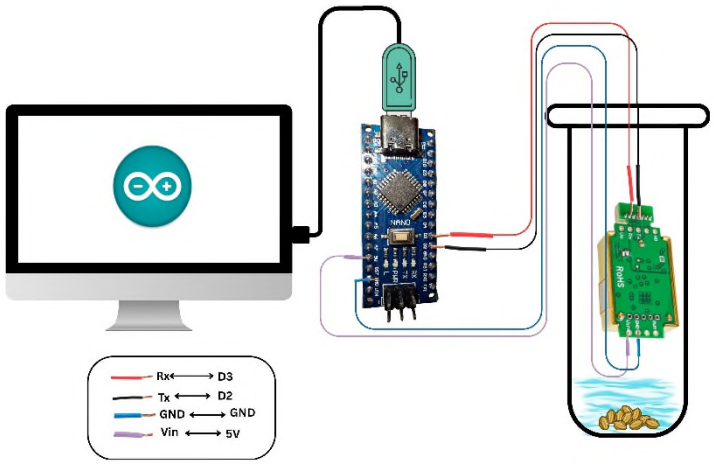


Fig. 2 Custom-built CO_2 sensor assemblies used for seed respiration measurement. The setup consists of an MH-Z19B CO_2 sensor connected to an Arduino Nano microcontroller and programmed using the Arduino IDE. The RX, TX, GND, and Vin pins of the sensor are connected to the D3, D2, GND, and 5V pins of the Nano, respectively. The Arduino Nano is powered through the USB supply from a computer. Seeds are placed in a 50 mL airtight tube containing the sensor to measure CO_2 evolution during incubation.

Germination and vigour assay

Standard germination tests were conducted following the International Rules for Seed Testing [31] with some modifications. For each crop, 50 seeds were placed in three replications using the between paper method (maize, wheat, green gram, soybean, pumpkin, cucumber) and top of paper method (broccoli). Germination counts were recorded at the final count, and the total percentage germination (GMAX, %) and total normal seedlings (TNS, %) were determined according to the ISTA Handbook on Seedling Evaluation [39]. From each replication, ten normal seedlings were randomly selected for measurement of shoot length and root length. These seedlings were subsequently dried in a hot-air oven at 70°C for 48 h to determine dry weight. Vigour indexes I and II were calculated according to Abdul-Baki and Anderson (1973) [40].

Preparation of bromothymol blue solution

Bromothymol blue (Sisco Research Laboratories Pvt. Ltd., India) was used at a concentration of 0.04 mg mL^{-1}, prepared by dissolving 40 mg of the BTB in 1000 mL distilled water. The pH of the solution was adjusted using NaOH. Based on responsiveness and clarity of colour change, pH 8.5 and 9.5 were selected, as higher pH values delayed visible colour transitions and reduced sensitivity. For viability assessment in large seeds like maize, wheat, green gram, soybean, pumpkin, and cucumber, pH 9.5 was used, and pH 8.5 for broccoli.

Standardization of soaking and incubation duration of the test using the BTB method

To standardize the soaking and incubation durations for viability testing, seeds from varieties exhibiting a germination percentage greater than 95% were selected for each crop. The seeds were soaked for varying time intervals (1 h, 2 h, 3 h, and 4 h) and subsequently incubated. During incubation, colour changes in the BTB solution were monitored at regular intervals. The optimal soaking and incubation durations were determined as the shortest time that produced colorimetric results closely corresponding to the actual germination percentage of each crop (Table 1).

Table 1. Soaking and incubation duration for seed viability estimation of seven crops

Crop	Soaking duration	Incubation duration
Maize	4 h	4 h
Wheat	4 h	5 h
Soybean	2 h	2.5 h
Green gram	2 h	2.5 h
Pumpkin	4 h	4 h
Cucumber	4 h	5 h
Broccoli	3 h	5 h

Seed viability testing using the BTB indicator

Seed viability was evaluated based on the colour change observed in the BTB solution. Pre-soaked seeds were placed in transparent 1.5 mL vials containing 250 µL of BTB solution adjusted to pH 8.5 or 9.5 (Table 1). The seeds were held in position using seed holders to prevent direct contact with the solution, and the vials were sealed to ensure airtight conditions (Fig. 3). A total of 150 seeds were tested in three replicates, and incubation was carried out at 25°C for different time intervals as indicated in Table 1. A colour shift of the indicator from blue to green or yellow during the incubation period was considered indicative of viable seeds, whereas the retention of the initial blue colour indicated non-viable seeds (Fig. 3).

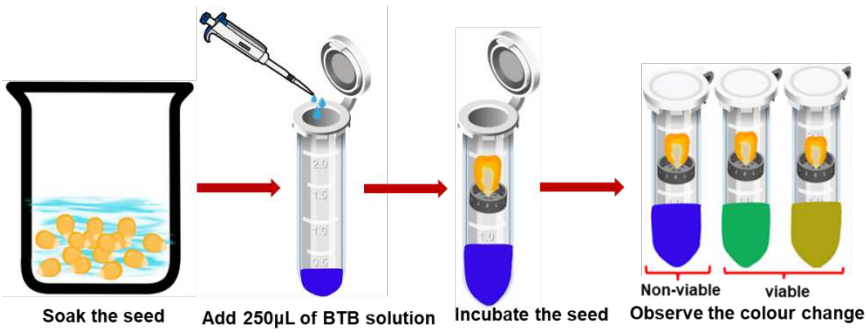


Fig. 3 Procedure for assessing seed viability using bromothymol blue (BTB). Seeds are first soaked in water for 2–4 h, followed by the addition of 250 µL of BTB solution (pH 8.5 or 9.5, depending on the crop) into a 1.5 mL vials. A seed holder is placed inside to secure the seed, and the tube is sealed airtight and incubated for 2.5–5 h. After incubation, non-viable seeds retain the initial blue colour of the indicator, while viable seeds cause a colour change to green or yellow.

Measurement of optical density of the BTB indicator solution.

The viability estimated by the BTB method was further validated spectrophotometrically by measuring the optical density (OD). Incubation vials containing viable seeds (green/yellow colour) exhibited an absorbance peak around 430-450 nm, while those with non-viable seeds (blue colour) showed a peak near 615 nm (Fig. 4). After completion of the incubation period, 220 µL of the BTB reagent was pipetted from each vial, and the OD was measured at 450 nm

using a microplate reader (Model BR-580, BR Biochem Life Sciences Pvt. Ltd., India) (Fig.5). The OD obtained was correlated with the total germination and with the vigour indices.

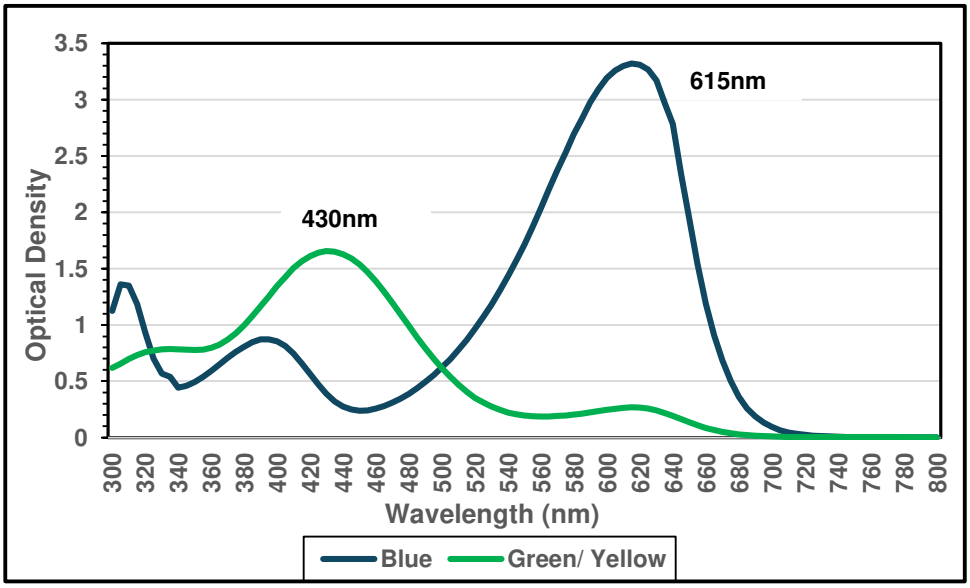


Fig. 4 Absorbance spectrum of bromothymol blue (BTB). The blue line represents the BTB solution at alkaline pH (> 7.6), while the green line represents the BTB solution at acidic pH (< 7.0).

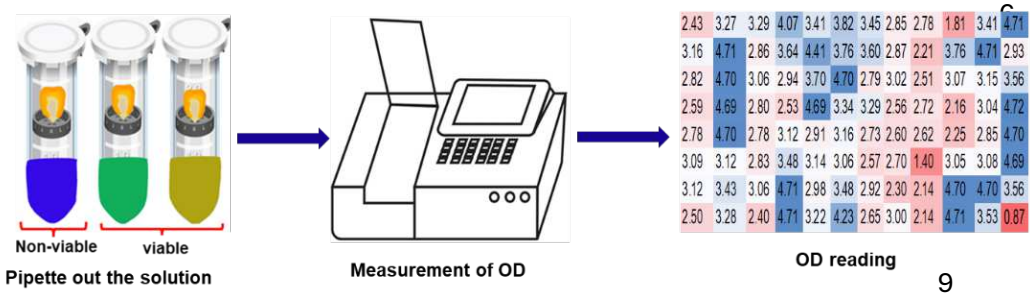


Fig. 5 Illustration for measuring optical density (OD) after incubation. After the completion of the incubation period, 200 µL of the bromothymol blue (BTB) solution is carefully pipetted out and transferred to a microplate well, and the OD is measured at 450 nm using a spectrophotometer.

Statistical analysis

Data collected for all parameters were subjected to statistical analysis using RStudio (version 10) and SAS software (version 9.4). The Least Significant Difference (LSD) values were computed for mean comparison. Contrast analysis using matrix functions in RStudio was performed instead of a multifactorial CRD analysis, and variance ratios were tested for

significance at the 5% probability level. Repeated-measures ANOVA was carried out using the General Linear Model (PROC GLM) procedure in SAS (version 9.4). The area under the curve (AUC) was calculated using R software, and treatment means were compared at a 5% level of significance. Percentage germination data were transformed to arcsine values prior to analysis.

Results

Comparative assessment of seed respiration in different crops

Seed respiration rates in maize, wheat, green gram, soybean, pumpkin, cucumber, and broccoli were measured at 30-minute intervals for four hours. The CO_2 evolution increased progressively over the 4-hour period across all crops (Fig. 6). Among the tested seeds, green gram exhibited the highest CO_2 evolution (1876 ppm seed^{-1}), followed by soybean (824 ppm seed^{-1}). Maize (560 ppm seed^{-1}), pumpkin (539 ppm seed^{-1}), cucumber (525 ppm seed^{-1}), and wheat (532 ppm seed^{-1}) showed comparable levels, while broccoli recorded the lowest CO_2 evolution (481 ppm seed^{-1}).

Repeated measures ANOVA (Suppl. Table S1) supported these observations. The between-subject effects revealed a highly significant influence of crop on CO_2 evolution ($F = 107.24$, $P < 0.0001$). The within-subject effects showed that time had a significant effect on CO_2 evolution ($F = 164.34$, $P < 0.0001$) and the crop × time interaction was also significant ($F = 50.31$, $P < 0.0001$). These results confirm that respiration rates (CO_2 evolution) differ significantly among the crops over time.

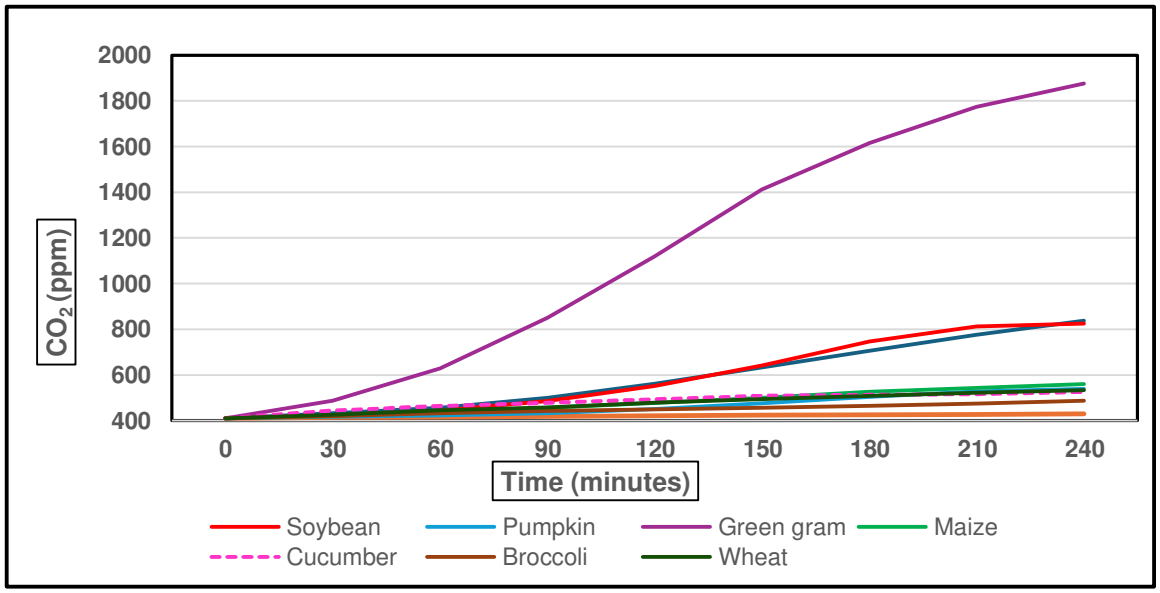


Fig. 6 CO_2 evolution per seed over time in different crop species. The line graph illustrates CO_2 release per seed measured for maize (n = 3), wheat (n = 5), soybean (n = 3), green gram (n = 3), broccoli (n = 10), pumpkin (n = 3), and cucumber (n = 3). An infrared CO_2 sensor was used to record the evolution of CO_2 for four hours. Measurements were recorded at 30 min intervals, and the rate of CO_2 evolution was expressed as the mean concentration in parts per million (ppm $seed^{-1}$).

To quantify respiration dynamics, the area under the curve (AUC) of CO_2 evolution over time was calculated using R software (Fig. 7). Significant differences were observed among crops, with the green gram showing the highest AUC (205159), followed by soybean (75974). The AUC values of cucumber and pumpkin were statistically similar, whereas broccoli recorded the lowest AUC (19582), confirming clear species-specific variation in respiration rates.

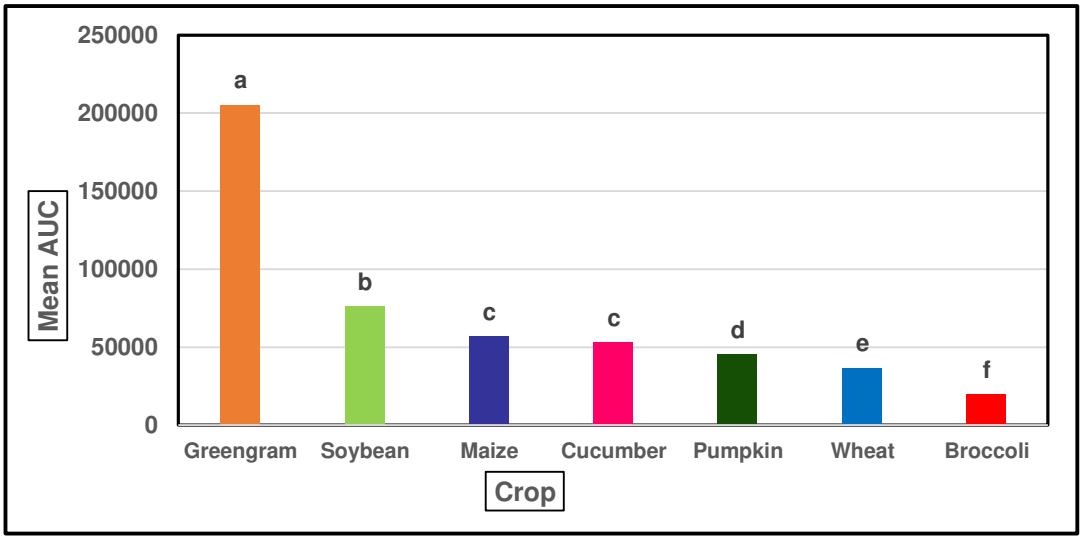


Fig. 7 Area under the curve (AUC) for CO_2 evolution by seeds of different crops—green gram, soybean, maize, cucumber, pumpkin, wheat, and broccoli. The AUC was calculated based on the trapezoidal integration method, which estimates total CO_2 release by summing the area between consecutive time points. Each bar represents the mean of three replicates (n = 3). Different letters denote significant differences among crops ($P < 0.05$).

Seed respiration in maize and soybean lots with different viability levels

In maize and soybean, different seed lots with varying germination percentages were evaluated. The CO_2 evolution rate was monitored using an infrared CO_2 sensor to assess how seed respiration relates to the physiological quality of each seed lot.

Seed respiration in maize seed lots with different viability levels

The respiration rate of three maize varieties: AGRA 2024, HKI-163, and IML-242-I, was measured over 4-h at 30 m intervals using an infrared CO_2 sensor. The germination percentages of these varieties were 100%, 84%, and 48%, respectively. The heatmap of CO_2 evolution over time (Fig. 8) showed a progressive increase in CO_2 release across all varieties, with colour intensity representing the magnitude of CO_2 evolution. Among them, AGRA 2024 exhibited the highest respiration rate (1053 ppm seed^{-1}), followed closely by HKI-163 (998 ppm seed^{-1}), while IML-242-I recorded the lowest rate (777 ppm seed^{-1}).

The repeated measures ANOVA (Suppl. Table S2) of CO_2 evolution in maize varieties across different time intervals revealed no statistically significant differences among varieties ($F = 2.80$, $P = 0.26$) or between replications ($F = 3.32$, $P = 0.21$). However, CO_2 evolution increased significantly over time, with a highly significant effect of time on respiration rate ($F = 73.38$, $P < 0.001$).

Similar trends were observed in the area under the curve (AUC) analysis (Fig. 9), where AGRA 2024 recorded highest AUC (183823.45) which was statistically similar to HKI-163. (180764), whereas lowest AUC values were recorded in IML-242-I (136764).

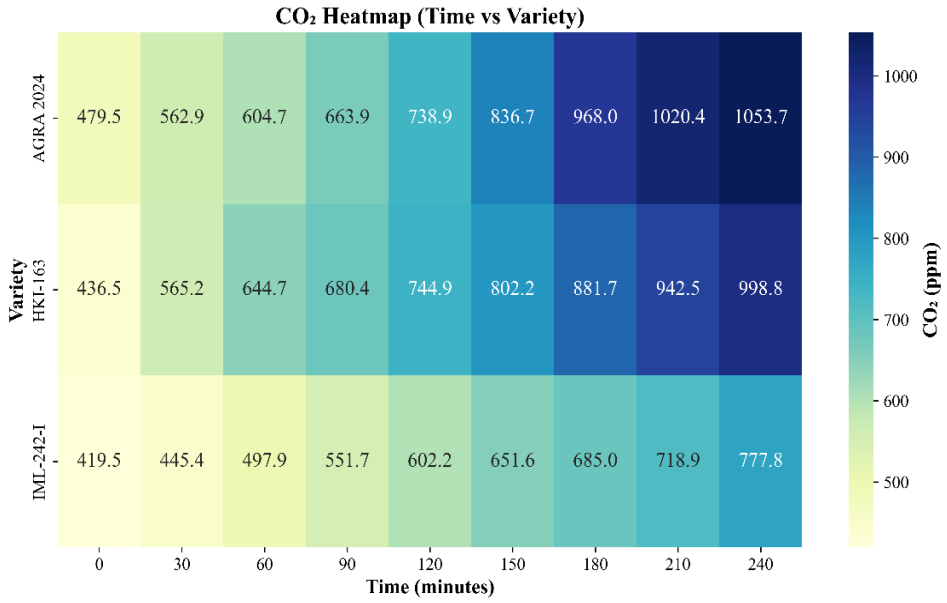


Fig. 8 Heatmap representation of CO_2 evolution in three maize varieties—AGRA (2024), HKI-163, and IML-242-I—recorded at sequential time intervals (0–240 minutes).

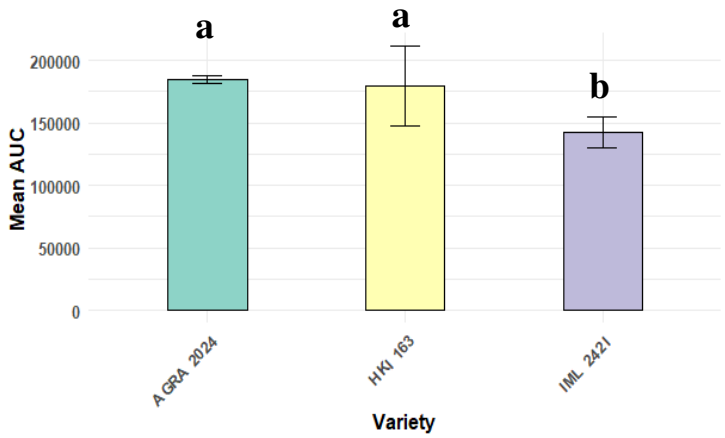


Fig. 9 Mean area under the curve (AUC) for CO_2 evolution in three maize varieties—AGRA (2024), HKI-163, and IML-242-I. The AUC represents the total CO_2 released during the incubation period, reflecting differences in seed metabolic activity and vigour. Each bar represents the mean of three replicates (n = 3), with vertical bars indicating standard error. Different letters denote significant differences among varieties ($P < 0.05$).

Seed respiration in maize seed lots with different viability levels

The respiration rate in the four soybean varieties namely SL 955, JS 2098, JS 9305, and JS 9560 having the germination percentage of 96%, 73%, 68%, and 48% respectively was measured over 3.5-h at 30 m intervals. The heatmap of CO_2 evolution (Fig. 10) revealed distinct varietal differences in CO_2 evolution. Variety SL 955 exhibited a steady increase in CO_2 evolution reaching the highest value of 1806 ppm seed^{-1} after 3.5-h of imbibition. Variety JS 0560 recorded lowest CO_2 lower evolution of 973 ppm seed^{-1}, while JS 2098 (1355 ppm seed^{-1}) and JS 9305 (1231 ppm seed^{-1}) displayed similar intermediate patterns over time. These results indicate that respiration rate, as reflected by CO_2 evolution, is positively associated with germination performance, with varieties possessing higher germination percentages exhibiting greater respiratory activity.

The repeated measures ANOVA (Suppl. Table S3) was conducted to evaluate the effects of variety, time, and replication on CO_2 evolution. The results revealed a non-significant difference among varieties for CO_2 evolution over time ($F = 5.88$, $P = 0.08$). However, CO_2

evolution increased significantly over time from 0 to 210 minutes (F = 121.98, $P < 0.0001$). The variety × time interaction was significant (F = 5.30, $P = 0.0002$). The interaction between variety and time also showed a statistically significant increase in CO_2 evolution as time progressed (F = 5.30, $P = 0.0002$), indicating that the rate of CO_2 evolution varied among varieties as time progressed.

The mean under curve (AUC) (Fig. 11) of these four varieties was calculated with the help of R software, where the variety SL 955 shows the highest area under curve (202068), followed by statistically similar area was exhibited by the variety JS 2098 (184480). and JS 9305 (146551). The lowest area occupied by the variety JS 9560 (134480).

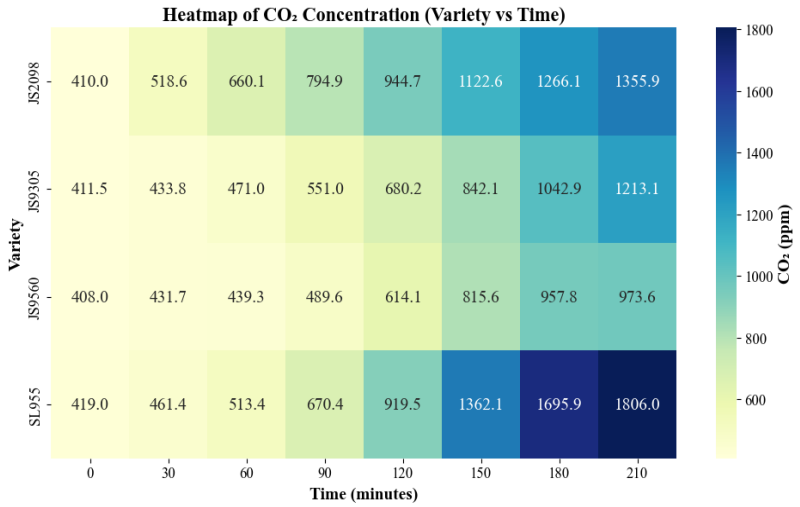


Fig. 10 Heatmap representation of CO_2 evolution in four soybean varieties— SL 955, JS 2098, JS 9305, and JS 9560—recorded at sequential time intervals (0–210 minutes).

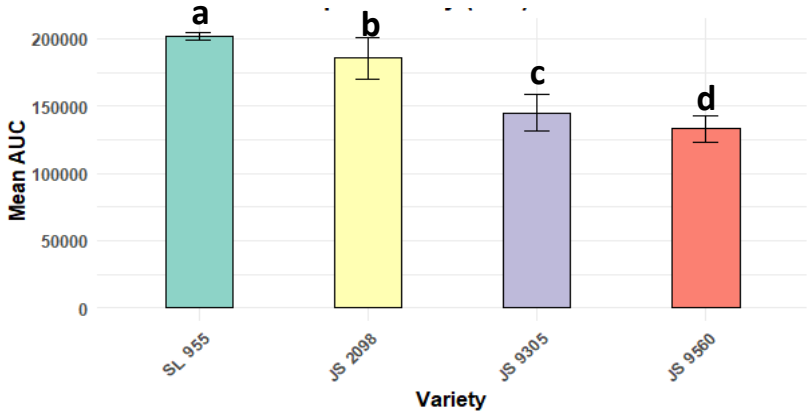


Fig. 11 Mean area under the curve (AUC) for CO_2 evolution in four soybean varieties— SL 955, JS 2098, JS 9305, and JS 9560. The AUC represents the total CO_2 released during the incubation period, reflecting differences in seed metabolic activity and vigour. Each bar represents the mean of three replicates (n = 3), with vertical bars indicating standard error. Different letters denote significant differences among varieties ($P < 0.05$).

Viability and vigour assessment by BTB method in maize seed lots.

The viability of six maize varieties was assessed using the standard germination test and compared with the bromothymol blue (BTB) indicator method (Table 2). For AGRA 2024, both methods recorded 100% germination, showing no deviation. In HKI 163, the BTB method estimated 88% viability compared to 85% actual germination, showing a 3% deviation. For PML 96, viability was slightly overestimated (70% vs. 65%), with a 5% deviation. In IML-242-I, the BTB method underestimated viability (39% vs. 48%), with the highest deviation of 8%. For IML-127-I, the deviation was minimal (21% vs. 23%, 2% deviation). In PML-92, having 0% total the BTB method recorded 1% germination with only a 1% deviation. For normal germination (seedling evaluation as per ISTA, 2025), the deviation was higher ranging from (2% to 31%).

ANOVA (Suppl. Table S4) showed significant variation among varieties for germination, while no significant difference was observed between the two germination assessment methods (total germination test and BTB method) (F=0.25, P=0.62). The high coefficient of determination

(R^2=0.98) indicated strong agreement between the methods, and the coefficient of variation (CV=8.5%) suggested low variability. In contrast, ANOVA (Suppl. Table S5) showed significant differences between normal germination (standard test) and viability estimated by the BTB method (F=31.57, P<0.0001), with R^2=0.96 and a higher CV of 16.4%. Indicating that BTB method can clearly determine the total germination in maize and not suitable for estimating the normal germination as per ISTA, 2025 [31].

Table 2. Comparison of germination percentage between standard germination test and estimation using acid base indicator in maize.

Varieties	Total G (%)	Percent germination	Estimated viability (%)[#]	Deviation from total G (%)	Deviation from (Percent germination)
AGRA 2024	100 (90)*	98 (81.87)	99 (84.26)	1	1
HKI 163	85 (66.94)	66 (54.33)	88 (69.73)	3	22
PML 96	65 (53.52)	39 (38.44)	70 (56.79)	5	31
IML 242-I	48 (43.85)	20 (26.56)	39 (38.84)	9	19
IML 127-I	23 (28.43)	9 (17.11)	21 (27.03)	2	12
PML 92	0 (0)	0 (0)	1 (5.74)	1	0

*Values in the parenthesis are arcsine-transformed values. Total G (%) includes all germinated seed. Percent germination is based on ISTA, 2025 criteria for seedling evaluation. [#]Viability estimated by BTB method.

Significant varietal differences were observed in vigour indices. AGRA 2024 recorded the highest vigour index I (2720) and vigour index II (51.20), whereas PML-92 showed the lowest vigour index I (0) and vigour index II (0) (Fig. 12 a & b).

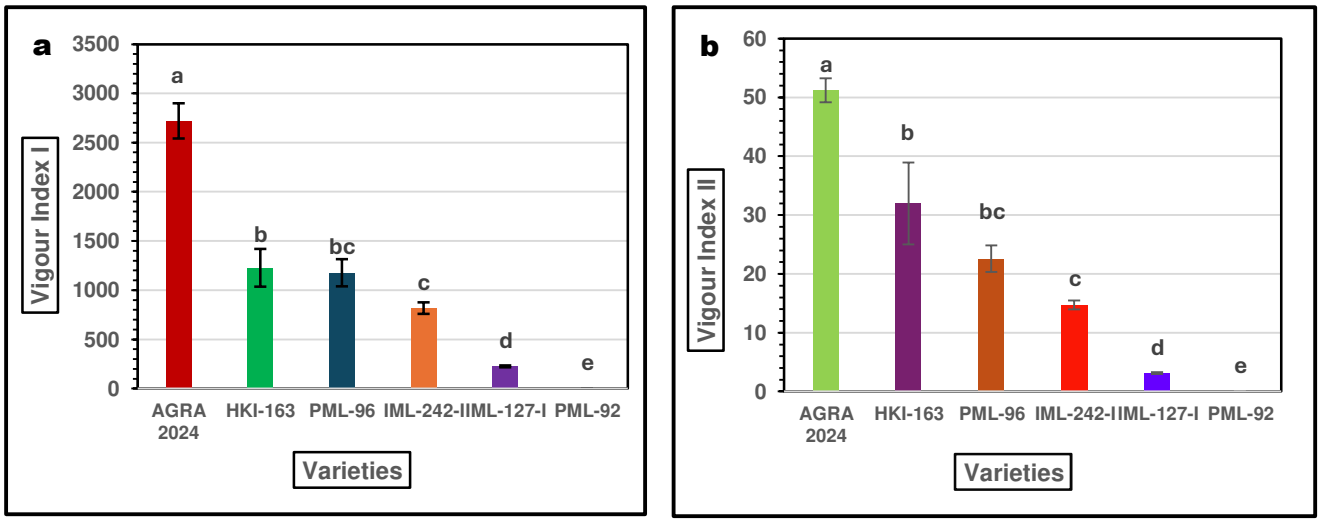


Fig. 12 Vigour indices of six maize seed lots—AGRA (2024), HKI-163, PML-96, IML-242-I, IML-247-I, and PML-92—showing (a) vigour index-I and (b) vigour index-II. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among seed lots ($P < 0.05$).

Optical density of BTB indicator in maize seed lots varying in germination and vigour

The viability of six maize varieties estimated by the BTB method was further validated using spectrophotometric analysis, wherein the optical density (OD) at 450 nm of the BTB indicator solution was measured after incubation. The OD values decreased proportionally with the decline in per cent germination (Fig. 13). Among the six maize varieties, AGRA 2024 recorded the highest OD value (2.48) which was statistically similar to HKI 163 (2.36), followed by PML-96 (2.09), while PML-92 exhibited the lowest OD value (1.03).

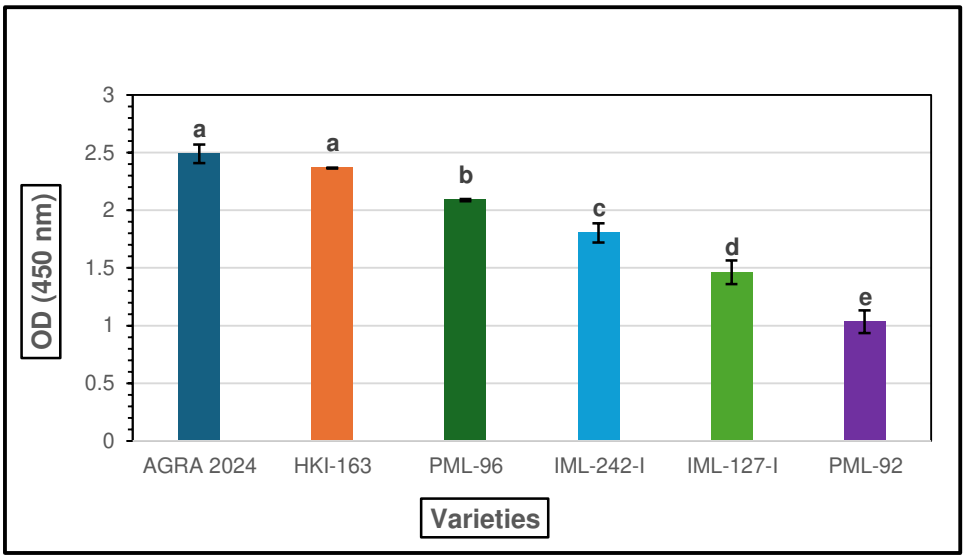


Fig. 13 Optical density (OD) values of bromothymol blue solution after incubation with seeds from six lots of maize varieties—AGRA 2024, HKI-163, PML-96, IML-242-I, IML-247-I and PML-92. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among seed lots ($P < 0.05$).

Linear regression (Fig. 14) plotted between total germination percentage and optical density, showed strong positive relationship with an r^2 of 0.99. Similarly, optical density exhibited significant correlations with vigour index-I ($r^2 = 0.78$) and vigour index-II ($r^2 = 0.87$) (Fig. 15 a & b).

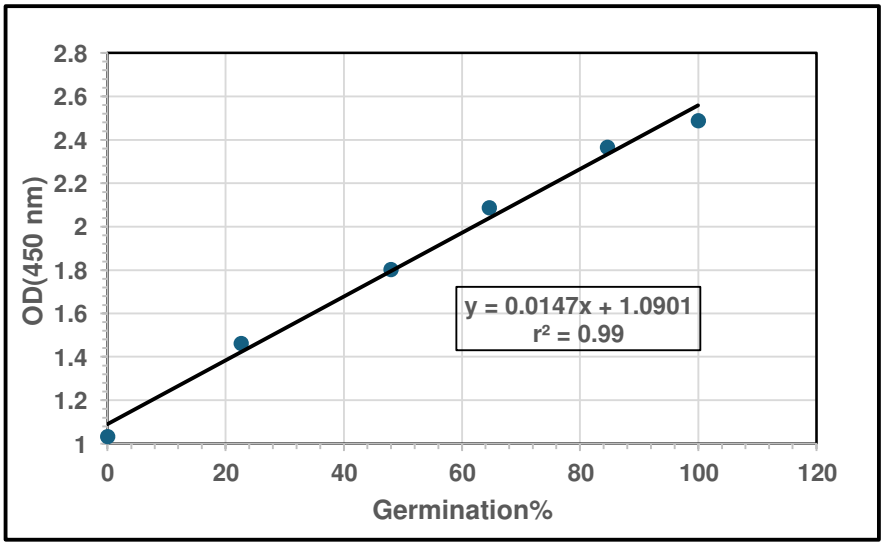


Fig. 14 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from six maize seed lots—AGRA (2024), HKI-163, PML-96, IML-242-I, IML-247-I, and PML-92—and their corresponding germination percentages. Each data point represents the mean of three replicates (n = 3).

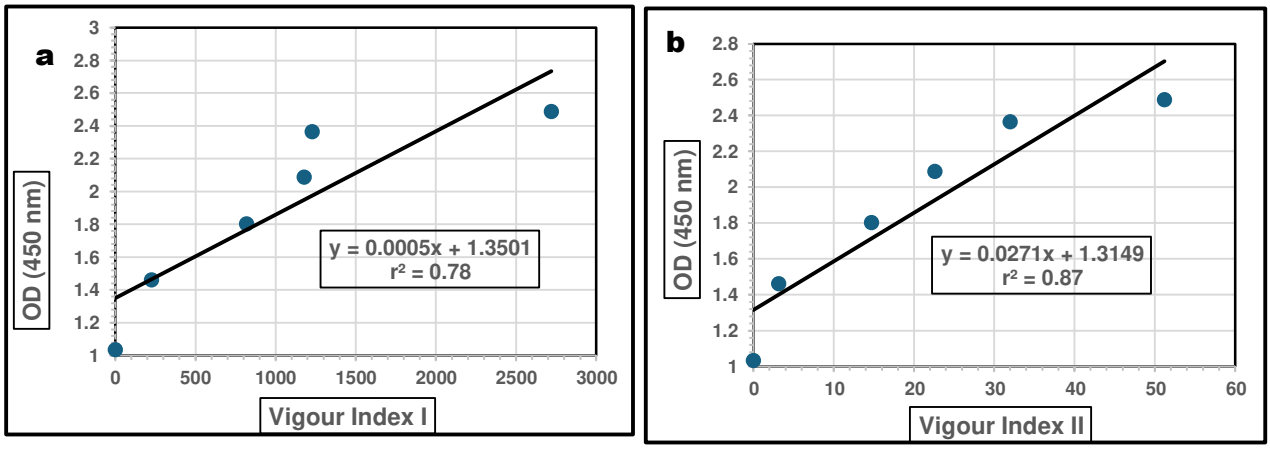


Fig. 15 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from six maize seed lots—AGRA (2024), HKI-163, PML-96, IML-242-I, IML-247-I, and PML-92—and their (a) vigour index-I and (b) vigour index-II. Each data point represents the mean of three replicates (n = 3).

Viability and vigour assessment by BTB method in wheat seed lots

The viability of three wheat varieties was evaluated using the standard germination test and compared with the bromothymol blue (BTB) indicator method (Table 3). For HD 3271, the actual germination percentage was 100%, while the BTB method estimated 93% viability, showing a 7% deviation from the standard germination test (ISTA, 2025). In PDW 215, actual germination was 79%, and estimated viability was 82%, with a 3% deviation. For the heat-killed HD 3271 seeds, actual germination was 0%, and the BTB method estimated 2% viability, showing a 2% deviation. For normal germination (seedling evaluation as per ISTA, 2025), the deviation was ranging from (3% to 9 %).

ANOVA (Suppl. Table S6) revealed significant variation among varieties for germination ($P < 0.0001$), while the differences between methods (total germination and the BTB method) were non-significant ($F = 0.13$, $P = 0.73$). The analysis showed a high coefficient of determination ($r^2 = 0.99$) and a low coefficient of variation (CV = 6.68%), indicating strong agreement between methods. Similarly, ANOVA (Suppl. Table S7) showed no significant difference between normal germination (standard test) and viability estimated by the BTB method ($F = 1.49$, $P = 0.24$), supported by an r^2 of 0.99 and a CV of 7.6%. These results indicate that the

BTB method can accurately assess both total and normal germination, in accordance with ISTA (2025) standards.

Table 3. Comparison of germination percentage between standard germination test and estimation using acid base indicator in wheat varieties.

Varieties	Total G (%)	Percent germination	Estimated viability (%)[#]	Deviation from total G (%)	Deviation from (Percent germination)
HD3271	100 (90)*	96 (78.46)	93 (75.03)	7	3
PDW 215	79 (62.49)	73 (58.91)	82 (64.64)	3	9
HD3271 (19-20)	0(0)	0(0)	2 (7.40)	2	0

*Values in the parenthesis are arcsine-transformed values. Total G (%) includes all germinated seed. Percent germination is based on ISTA, 2025 criteria for seedling evaluation. [#]Viability estimated by BTB method.

Significant varietal differences were observed in vigour indices. HD 3271 recorded the highest vigour index I (2308.33), followed by PDW 215 (814.7), whereas the heat-killed HD 3271 seeds showed zero values for both vigour index I and vigour index II (Fig. 16a). Similarly, vigour index II also varied significantly among varieties, ranging from 5.32 in PDW 215 to 8.24 in HD 3271 (Fig. 16b).

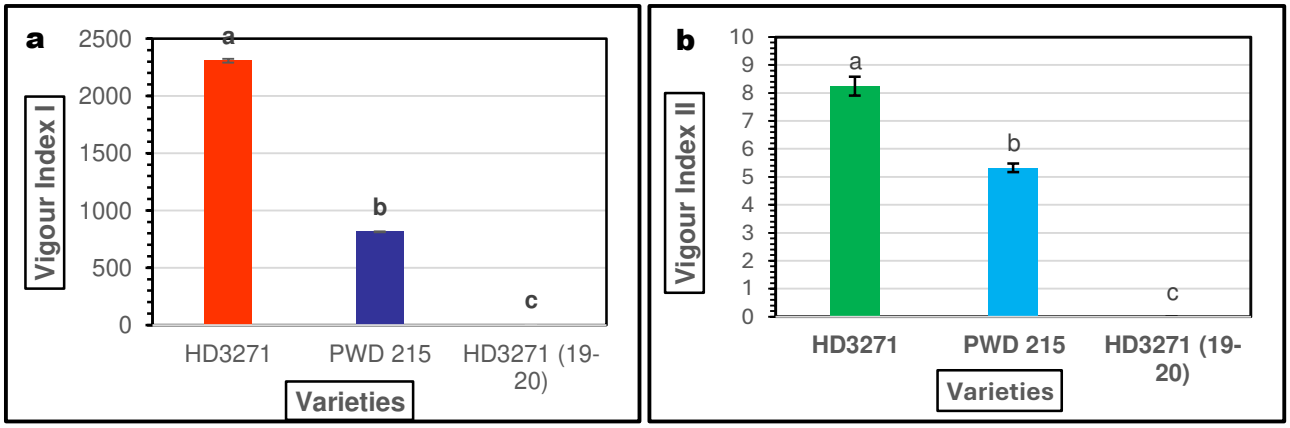


Fig. 16 Vigour indices of three wheat seed lots—HD 3271, PWD 216, and HD 3271 (2019–20)—showing (a) vigour index-I and (b) vigour index-II. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among seed lots ($P < 0.05$).

Optical density of BTB indicator in wheat seed lots varying in germination and vigour

The viability of three wheat varieties estimated by the BTB method was validated through spectrophotometric analysis, wherein the optical density (OD) at 450 nm of the BTB indicator solution was measured after incubation. The OD values decreased proportionally with the decline in germination percentage (Fig. 17). The variety HD 3271, which showed the highest germination (100%), recorded an OD value of 1.51, followed by PDW 215 with an OD value of 1.28. In contrast, the heat-killed seed lot of HD 3271 (19–20) exhibited the lowest OD value (0.87).

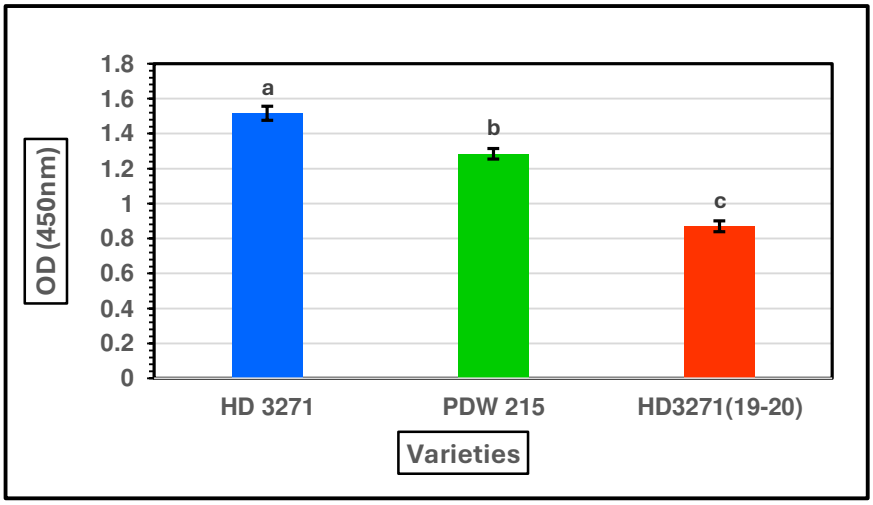


Fig. 17 Optical density (OD) values of bromothymol blue solution after incubation with seeds from three wheat seed lots—HD 3271, PWD 216, and HD 3271 (2019–20). Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among seed lots *(P* < 0.05).

Linear regression (Fig. 18) revealed strong positive relationship between total germination percentage and optical density, with an r^2 of 0.98. Similarly, regression analysis of optical density with vigour index-I and vigour index-II showed significant correlations, with r^2 values of 0.89 and 1.0, respectively (Fig. 19 a & b).

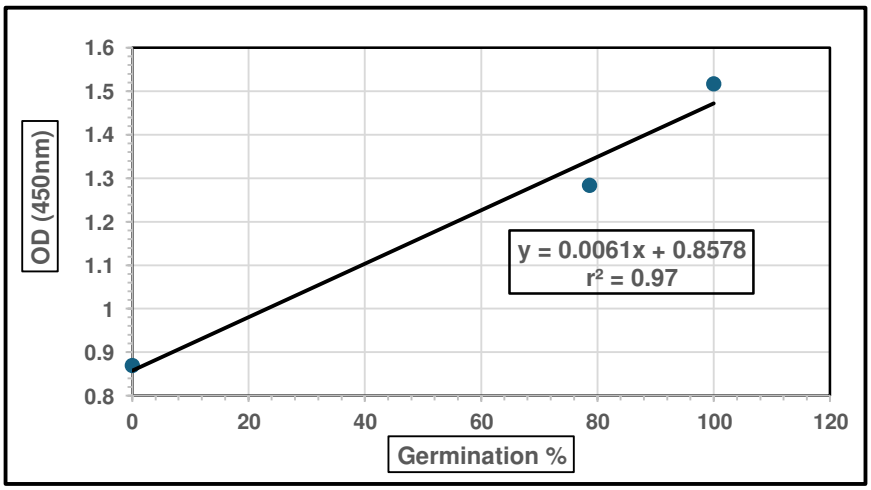


Fig. 18 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from three wheat seed lots—HD 3271, PWD 216, and HD 3271 (2019–20)—and their corresponding germination percentages. Each data point represents the mean of three replicates (n = 3)

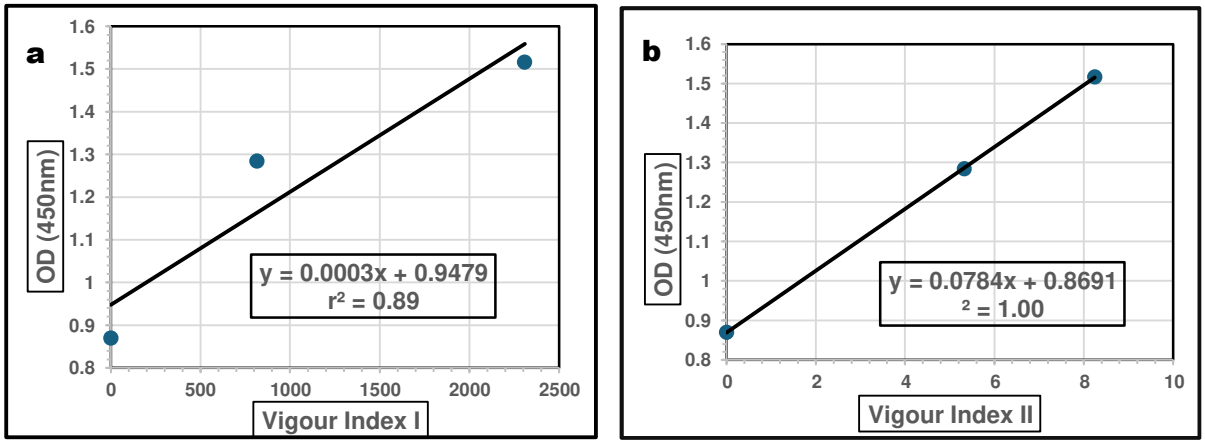


Fig. 19 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from three wheat seed lots—HD 3271, PWD 216, and HD 3271 (2019–20)—and their (a) vigour index-I and (b) vigour index-II. Each data point represents the mean of three replicates (n = 3).

Viability and vigour assessment by BTB method in soybean seed lots.

The viability of four varieties of soybean estimated by standard germination test and by using BTB Method (Table 4). For the variety SL 955 having the actual germination percentage was 96%, the estimated viability by the BTB method was also 91% with 5% deviation from the standard germination test. For variety JS 2098 & JS 9305 having the actual germination percentage of 73% and 68% respectively, where the estimated viability percentage by BTB method was 73% and 63% respectively with 5% deviation from the standard germination test. The variety JS 9560 having the actual germination of 48%, the estimated viability by the BTB method was 56% with the deviation of 8% deviation from the standard germination test. For normal germination (seedling evaluation as per ISTA, 2025), the deviation ranged from (11% to 31 %).

ANOVA (Suppl. Table S8) shows the significant variation among varieties for germination, while differences between methods (total germination by standard test vs. BTB) were non-significant (F = 0.12, *P* = 0.73), with high r² (0.92) and low CV (7.5%). In contrast, ANOVA (Suppl. Table S9) revealed significant differences between normal germination (standard test)

and BTB-estimated viability (F = 8.28, P = 0.003), with an r^2 of 0.86 and a CV of 14.29%. Indicating that BTB method can clearly determine the total germination and not suitable for estimating the normal germination as per ISTA, 2025.

Table 4. Comparison of germination percentage between standard germination test and estimation using BTB method in soybean varieties.

Varieties	Total G (%)	Percent germination	Estimated viability (%)[#]	Deviation from total G (%)	Deviation from (Percent germination)
SL 955	96 (78.46)*	80 (63.91)	91 (72.875)	5	11
JS 2098	73 (58.91)	60 (50.77)	73 (58.91)	0	13
JS 9305	68 (55.55)	38 (38.06)	63(52.73)	5	25
JS 9560	48 (48.85)	25(30.22)	56 (48.45)	8	31

*Values in the parenthesis are arcsine-transformed values. Total G (%) includes all germinated seed. Percent germination is based on ISTA, 2025 criteria for seedling evaluation. [#]Viability estimated by BTB method.

Significant varietal differences were observed in vigour indices. Vigour index-I varied significantly, ranging from 1069.95 in JS 9560 to 3679.33 in SL 955 (Fig. 20a). Similarly, vigour index-II was highest in SL 955 (326.27) and lowest in JS 9560 (129.20), with significant differences among the varieties (Fig. 20b).

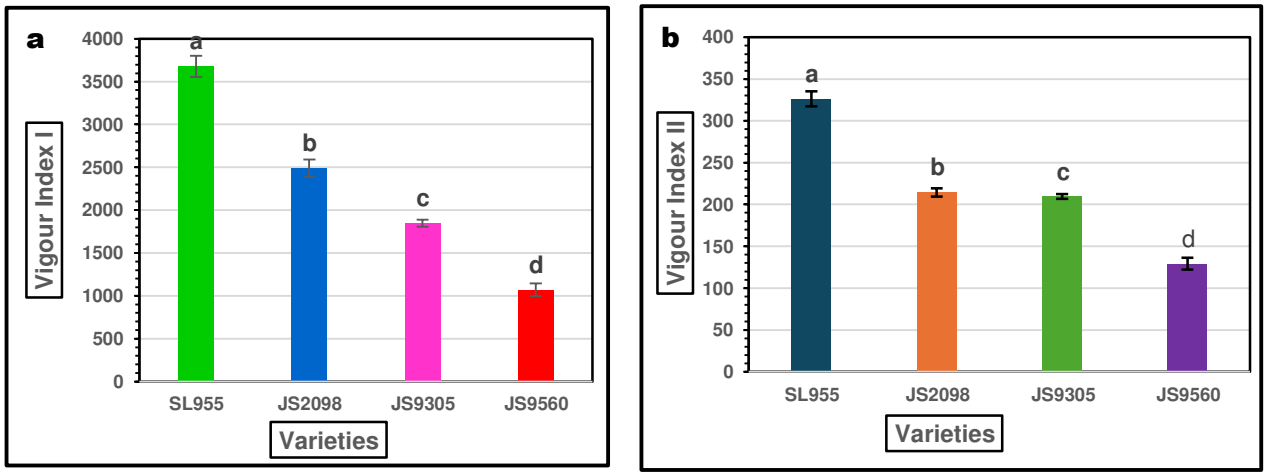


Fig. 20 Vigour indices of four soybean varieties—SL 955, JS 2098, JS 9305, and JS 9560—showing (a) vigour index-I and (b) vigour index-II. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among varieties ($P < 0.05$).

Optical density of BTB indicator in soybean seed lots varying in germination and vigour

The viability of the four soybean varieties estimated by the BTB method was validated through spectrophotometric analysis, where the optical density (OD) was measured at 450 nm. A significant positive correlation ($P \leq 0.01$) was observed between percent germination and seed viability (Fig. 21). The OD value for variety SL 955 having the germination of 96% observed was 2.61**,** while that for variety JS 2098 & JS 9305 was 2.44 & 2.39 respectively. Similarly for the variety JS 9560 having the lowest germination (48%) among the varieties showed the OD values of 2.19.

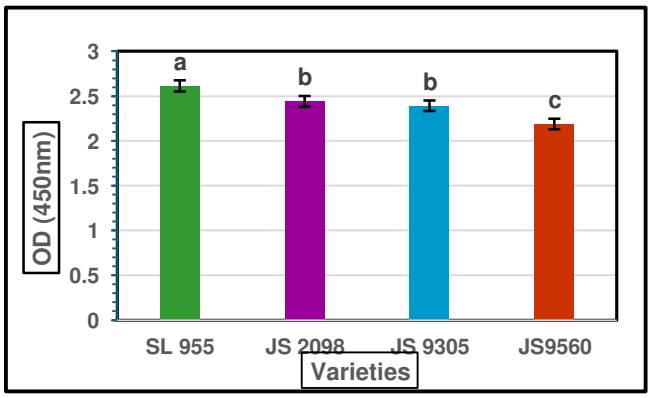


Fig. 21 Optical density (OD) values of bromothymol blue solution after incubation with seeds from four soybean varieties—SL 955, JS 2098, JS 9305, and JS 9560—following the completion of the incubation period. Each bar represents the mean of three replicates (n = 3).

Vertical bars indicate standard error. Different letters denote significant differences among varieties ($P < 0.05$).

Linear regression (Fig. 22) revealed strong positive relationship between total germination percentage and optical density, with an r^2 of 0.94. Similarly, regression analysis of optical density with vigour index-I and vigour index-II showed significant correlations, with r^2 values of 0.86 and 0.96 respectively (Fig. 23 a & b).

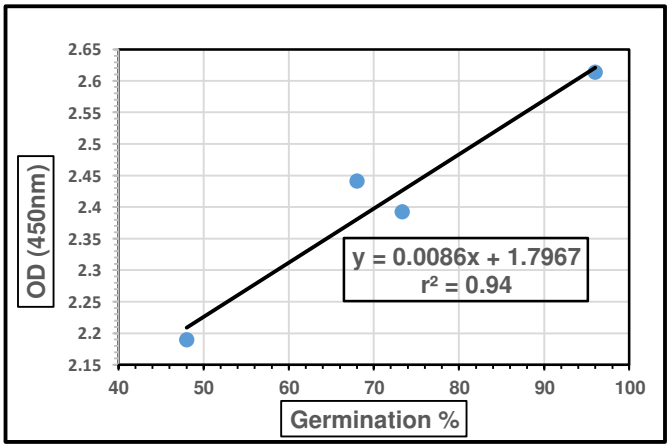


Fig. 22 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from four soybean varieties—SL 955, JS 2098, JS 9305, and JS 9560—and their corresponding germination percentages. Each data point represents the mean of three replicates (n = 3).

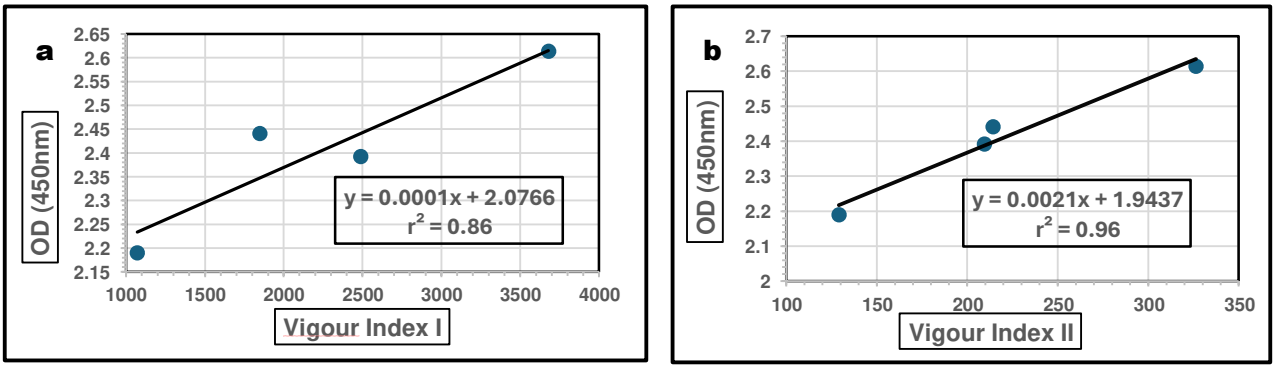


Fig. 23 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from four soybean varieties—SL 955, JS 2098, JS 9305, and JS 9560—and their (a) vigour index-I and (b) vigour index-II. Each data point represents the mean of three replicates (n = 3).

Viability and vigour assessment by BTB method in green gram seed lots

The viability of two green gram varieties, as determined by the standard germination test, was compared with the bromothymol blue (BTB) indicator method (Table 5). For the variety Pusa 1641 (SM 2024), which had an actual germination of 99%, the BTB-estimated viability was 93%, showing a deviation of 6%. For the variety Pusa 1641 (KH 2021), which had an actual germination of 61%, the BTB method estimated viability at 63%, with a deviation of 2% from the standard germination test. For normal seedling evaluation (as per ISTA, 2025), the deviation ranged from 8% to 9%.

ANOVA (Suppl. Table S10) showed significant variation among varieties for total germination, while differences between the two methods (total germination vs. BTB-estimated viability) were non-significant ($F = 0.51$, $P = 0.62$). The analysis also showed a high r^2 (0.96) and a low CV (5.4%), indicating strong agreement between the methods for estimating total germination. In contrast, ANOVA (Suppl. Table S11) revealed significant differences between normal germination (standard seedling evaluation) and BTB-estimated viability, with a non-significant F value ($F = 0.28$, $P = 0.76$), an r^2 of 0.86, and a CV of 6.42%. These results indicate that the BTB method reliably estimates total germination but is not suitable for assessing normal germination as defined by ISTA (2025) seedling evaluation criteria.

Table 5. Comparison of germination percentage between standard germination test and estimation using BTB method in green gram varieties.

Varieties	Total G (%)	Percent germination	Estimated viability (%)[#]	Deviation from total G (%)	Deviation from (Percent germination)
Pusa 1641 (SM 2024)	99 (83.35)*	85 (67.48)	93 (74.28)	6	8
Pusa 1641 (KH 2021)	61 (51.55)	54 (47.29)	63 (52.33)	2	9

*Values in the parenthesis are arcsine-transformed values. Total G (%) includes all germinated seed. Percent germination is based on ISTA, 2025 criteria for seedling evaluation. [#]Viability estimated by BTB method.

Significant varietal differences were observed for two different lots green gram varietie Pusa 1641. Vigour Index-I showed a clear and significant variation, with Pusa 1641 (SM 2024) recording 2443.90 units, while Pusa 1641 (KH 2021) recorded 1165.30 units (Fig. 24a). Similarly, Vigour Index-II also differed significantly, with Pusa 1641 (SM 2024) showing 21.14 units compared to 11.48 units in Pusa 1641 (KH 2021) (Fig. 24b).

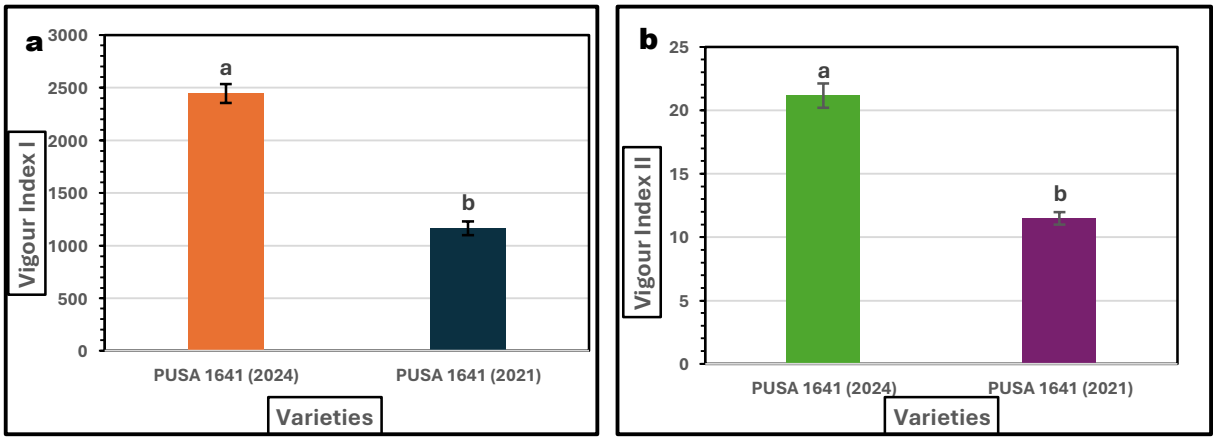


Fig. 24 Vigour indices of two green gram seed lots—Pusa 1641 (SM 2024) and Pusa 1641 (KH 2021)—showing (a) vigour index-I and (b) vigour index-II. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences between seed lots ($P < 0.05$). Viability assessment by using BTB method in cucumber seed varieties.

Optical density of BTB indicator in green gram seed lots varying in germination and vigour

The viability of the two green gram varieties estimated by the BTB method was validated through spectrophotometric analysis, where the optical density (OD) was measured at 450 nm after the completion of the incubation period. A significant positive correlation ($P \leq 0.01$) was observed between percent germination and seed viability (Fig. 25). The OD value for variety Pusa 1641(SM 2024) having the germination of 99% was 2.49, while that for variety Pusa 1641 (KH 2021) having the germination percentage of 61% was 2.06.

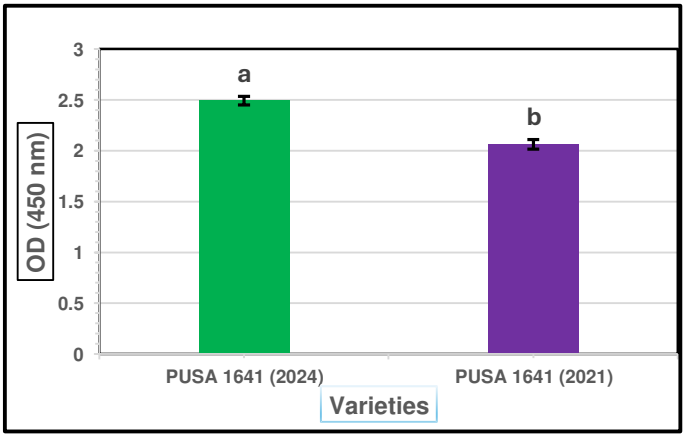


Fig. 25 Optical density (OD) values of bromothymol blue solution after incubation with seeds from two green gram seed lots—Pusa 1641 (SM 2024) and Pusa 1641 (KH 2021)—following the completion of the incubation period. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences between seed lots ($P < 0.05$).

Viability and vigour assessment by BTB method in cucumber seed lots

The viability of five varieties of cucumber estimated by standard germination test was compared using the bromothymol blue indicator method (BTB Method) (Table 6). For the variety Avani (2024) having the actual germination percentage was 92%, the estimated viability by the BTB method was 88% with 4% deviation from the standard germination test. For variety Japanese Long Green (2022), Japanese Long Green (2021), Japanese Long Green (2018)

having the germination percentages of 85%, 78% & 64% respectively, where the estimated viability by BTB method were 86%,75% & 59% respectively with the deviation of 1%, 3%, 5% respectively from the standard germination test. The variety Japanese Long Green (2013) having 59%, the estimated viability by the BTB method was 65% with the deviation of 6% from the standard germination test. For normal germination (seedling evaluation as per ISTA, 2025), the deviation was ranging from (0% to 38%).

ANOVA (Suppl. Table S12) revealed significant variation among cucumber varieties for germination, whereas non-significant difference was observed between the germination methods (total germination by standard test vs. BTB method; $F=1.18$, $P=0.29$) with r^2 value of 0.97 and CV=3.5%. In contrast, ANOVA (Suppl. Table S13) showed significant differences between normal seedlings assessed by the standard test and viability estimated by the BTB method ($F=30.09$, $P<0.001$), with r^2 value of 0.88 and CV=11.2%. Indicating that BTB method can clearly determine the total germination and not suitable for estimating the normal germination as per ISTA, 2025.

Table 6. Comparison of germination percentage between standard germination test and estimation using BTB method in cucumber varieties.

Varieties	Total G (%)	Percent germination	Estimated viability (%)[#]	Deviation from total G (%)	Deviation from (Percent germination)
Avani (2024)	93 (74.28)*	88 (69.73)	88 (69.73)	5	0
Japanese Long Green (2022)	78 (62.02)	69 (56.37)	75 (60.21)	3	6
Japanese Long Green (2021)	85 (66.94)	65 (53.92)	86 (68.30)	2	21
Japanese Long Green (2018)	64 (52.92)	45 (41.74)	59 (50.18)	5	14
Japanese Long Green (2013)	59 (49.98)	27 (31.08)	65 (53.75)	6	38

*Values in the parenthesis are arcsine-transformed values. Total G (%) includes all germinated seed. Percent germination is based on ISTA, 2025 criteria for seedling evaluation. [#]Viability estimated by BTB method.

The vigour index varied significantly among the varieties, highest vigour index-I was recorded in Avani (2024) having 2096.57 units and Japanese Long Green (2013) recorded lowest value of 690.20 units (Fig. 26a). A similar trend was observed for vigour index-II, where Avani (2024) exhibited the maximum of 7.89 units, while Japanese Long Green (2013) recorded the minimum of 2.93 units (Fig. 26b).

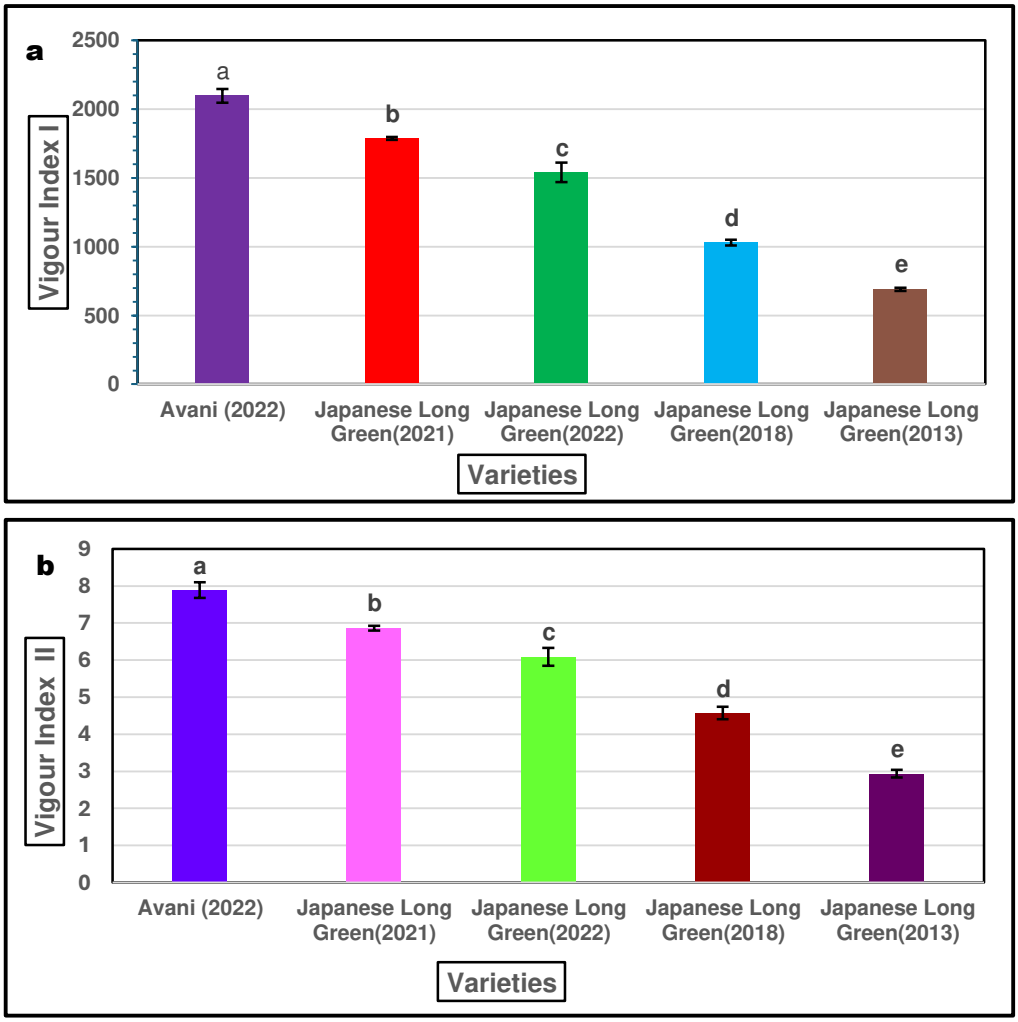


Fig. 26 Vigour indices of six cucumber seed lots—Avani (2022) and Japanese Long Green (2022, 2021, 2018, 2013, and 2011)—showing (a) vigour index-I and (b) vigour index-II. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among seed lots ($P < 0.05$).

Optical density of BTB indicator in cucumber seed lots varying in germination and vigour

The viability of the five cucumber varieties estimated by the BTB method was validated through spectrophotometric analysis, where the optical density (OD) was measured at 450 nm after the completion of incubation period. A significant positive correlation ($P \leq 0.01$) was observed between percent germination and seed viability (Fig. 27). The OD value for variety Avani (2024) having the germination of 92% was 1.88, while that for variety Japanese Long Green (2022), Japanese Long Green (2021), Japanese Long Green (2018) were 1.78, 1.78, 1.56

respectively**.** Similarly for the variety Japanese Long Green (2013) having the lowest germination (59%) among the varieties recorded the lowest OD values of 1.50.

Linear regression (Fig. 28) revealed a strong positive relationship between total germination percentage and optical density with R^2=0.96. Similarly, regression analysis between optical density and vigour index-I (R^2=0.96) and vigour index-II (R^2=0.94) indicated strong significant correlations (Fig. 29 a & b).

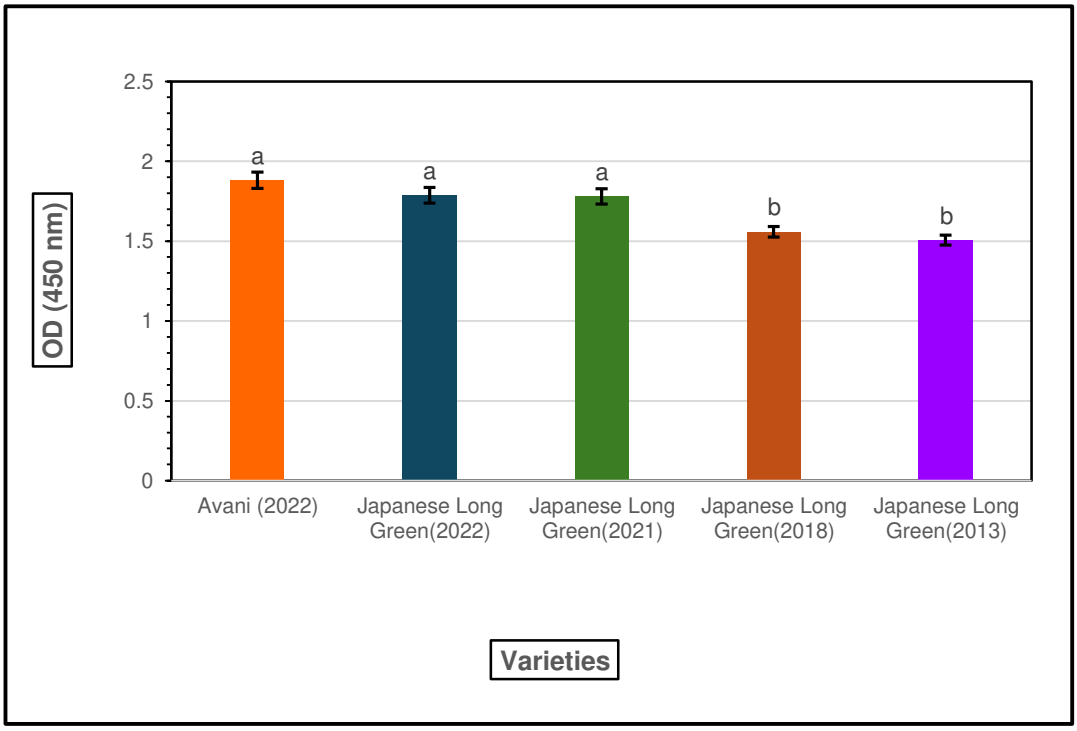


Fig. 27 Optical density (OD) values of bromothymol blue solution after incubation with seeds from six cucumber seed lots—Avani (2022) and Japanese Long Green (2022, 2021, 2018, 2013, and 2011)—following the completion of the incubation period. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among seed lots ($P < 0.05$).

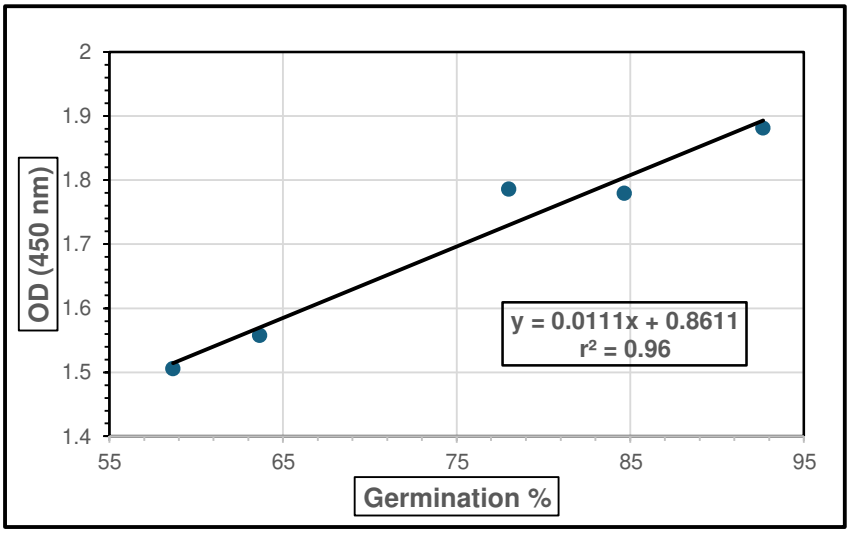


Fig. 28 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from six cucumber seed lots—Avani (2022) and Japanese Long Green (2022, 2021, 2018, 2013, and 2011)—and their corresponding germination percentages. Each data point represents the mean of three replicates (n = 3).

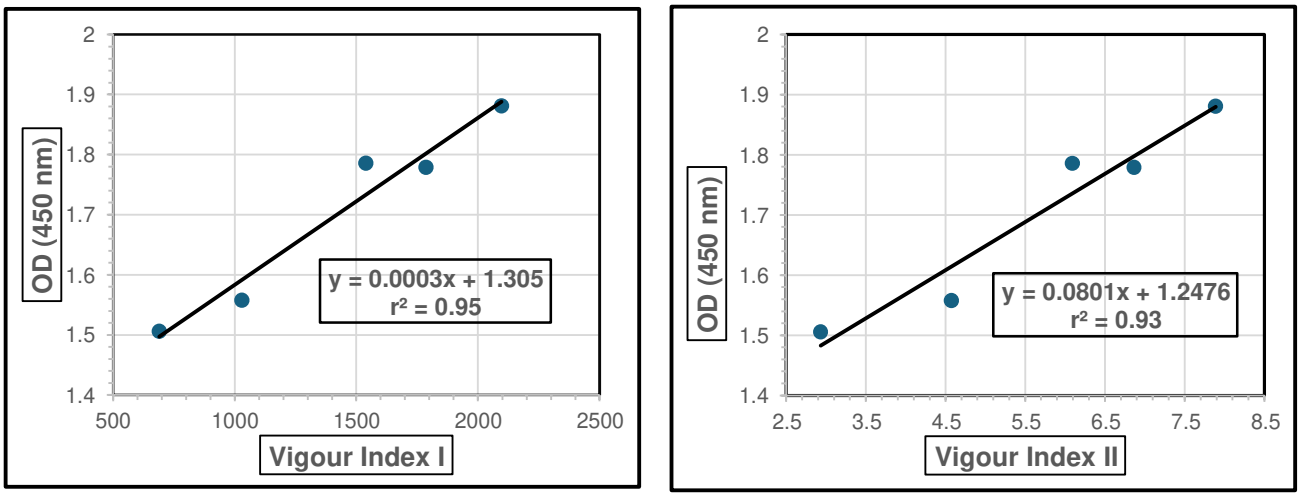


Fig. 29 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from five cucumber seed lots—Avani (2022) and Japanese Long Green (2022, 2021, 2018, and 2013)—and their (a) vigour index-I and (b) vigour index-II. Each point represents the mean OD and corresponding mean germination value of three replicates (n = 3).

Viability and vigour assessment by BTB method in broccoli seed lots

The viability of four seed lots of the variety Pusa Purple was estimated using both the standard germination test and the BTB method (Table 7). For Pusa Purple (2024), the actual germination

percentage was 89%, while the BTB method estimated viability was 85%, showing a 4% deviation. For the 2023 seed lot, the actual germination was 64%, and the BTB method estimated 69% viability, with a 5% deviation. In Pusa Purple (2021), the actual germination was 22%, the estimated viability was 20%, and the deviation was 2%. The oldest lot, Pusa Purple (2019), recorded 0% actual germination, whereas the BTB-estimated viability was 3%, resulting in a 3% deviation. For normal germination (seedling evaluation as per ISTA, 2025), the deviations ranged from 0% to 22%.

ANOVA (Suppl. Table S14) revealed significant variation among varieties for germination, while no significant difference was observed between the two germination assessment methods (total germination vs. the BTB method; $F = 0.02$, $P = 0.88$), with an r^2 value of 0.99 and CV = 6.02%. In contrast, ANOVA (Suppl. Table S15) showed significant differences between normal seedling counts assessed by the standard test and the BTB method ($F = 14.9$, $P < 0.01$), with an r^2 value of 0.98 and CV = 14.57%. These results indicate that while the BTB method can reliably determine total germination, it is not suitable for estimating normal germination as per ISTA (2025) rules.

Table 7. Comparison of germination percentage between standard germination test and estimation using acid base indicator in broccoli.

Varieties	Total G (%)	Percent germination	Estimated viability (%)[#]	Deviation from total G (%)	Deviation from (Percent germination)
Pusa Purple (2024)	89 (70.93)*	75(60.21)	85 (66.94)	5	10
Pusa Purple (2023)	64 (53.32)	47 (43.28)	69 (55.95)	5	22
Pusa Purple (2021)	22 (27.73)	10 (18.43)	21 (27.03)	1	11
Pusa Purple (2019)	0 (0)	0 (0)	3 (10.51)	3	0

*Values in the parenthesis are arcsine-transformed values. Total G (%) includes all germinated seed. Percent germination is based on ISTA, 2025 criteria for seedling evaluation. [#]Viability estimated by BTB method.

Significant differences were observed in the vigour indices among the different seed lots of Pusa Purple. The 2024 lot recorded the highest vigour index-I (878.39) and vigour index-II (2.49), whereas the 2019 lot showed the lowest values, with both vigour index-I and II measuring 0 (Fig. 30 a & b).

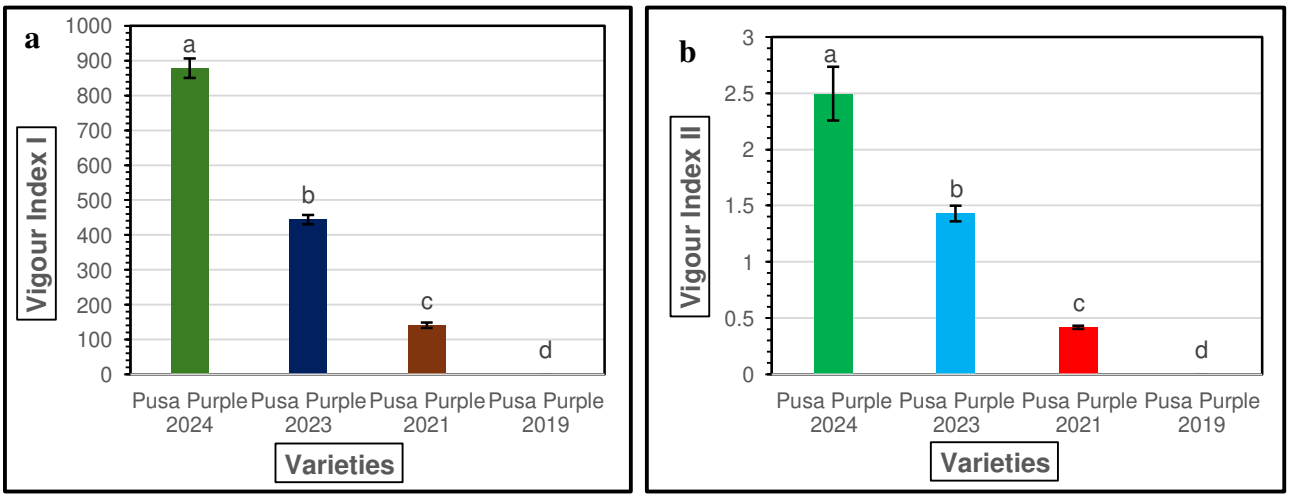


Fig. 30 Vigour indices of four seed lots of broccoli variety Pusa Purple: (a) vigour index-I and (b) vigour index-II. Each bar represents the mean of three replicates (n = 3). Vertical bars

indicate standard error. Different letters denote significant differences among seed lots ($P < 0.05$).

Optical density of BTB indicator in broccoli seed lots varying in germination and vigour

The viability of the four seed lots of Pusa Purple estimated using the bromothymol blue (BTB) indicator solution was further validated through spectrophotometric analysis. The optical density (OD) of the indicator solution measured at 450 nm after incubation showed a significant correlation with viability ($P < 0.001$) (Fig. 31). The OD values for Pusa Purple (2024), Pusa Purple (2023), Pusa Purple (2021), and Pusa Purple (2019) were 2.21, 1.64, 1.31, and 1.29, respectively, indicating that seed lots with higher viability exhibited correspondingly higher OD values.

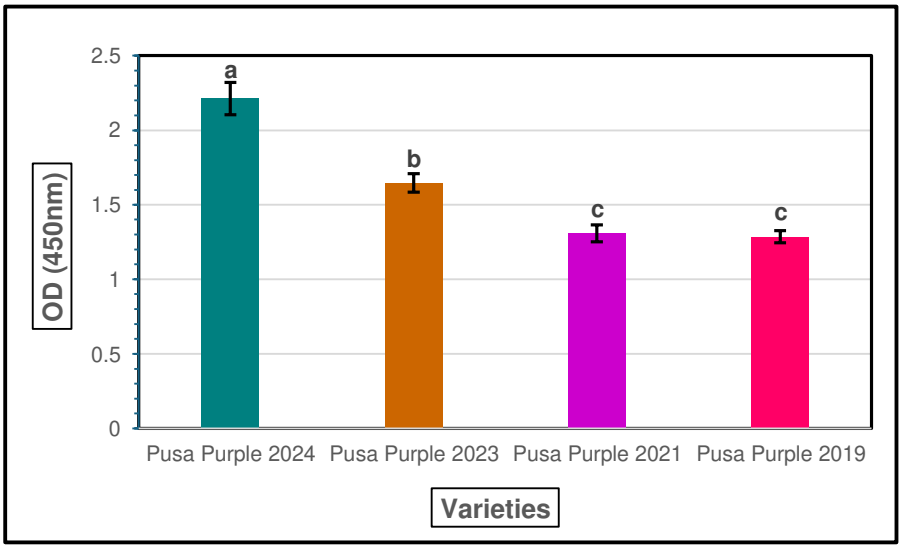


Fig. 31 Optical density (OD) values of bromothymol blue solution after incubation with seeds from four lots of broccoli variety Pusa Purple (2024, 2023, 2022, and 2021). Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences among seed lots ($P < 0.05$).

Linear regression analysis (Fig. 32) revealed a strong positive relationship between total germination percentage and OD ($r^2 = 0.87$). Similarly, regression analysis between OD and vigour index-I ($r^2 = 0.98$) and vigour index-II ($r^2 = 0.96$) showed strong and significant correlations (Fig. 33 a & b).

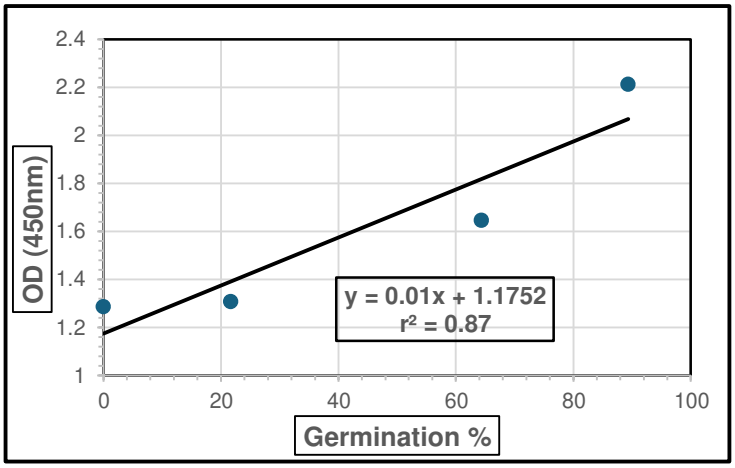


Fig. 32 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from four lots of broccoli variety Pusa Purple and their germination percentage. Each point represents the mean OD and corresponding mean germination value of three replicates (n = 3).

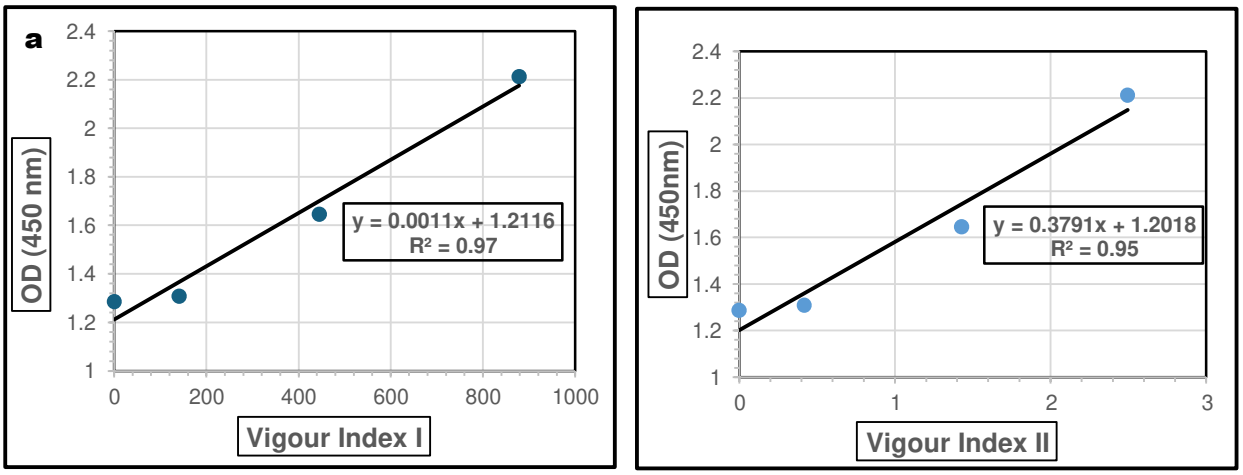


Fig. 33 Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation with seeds from four lots of broccoli variety Pusa Purple and their (a) vigour index-I and (b) vigour index-II. Each point represents the mean OD and corresponding mean vigour index value of three replicates (n = 3).

Viability and vigour assessment by BTB method in pumpkin seed lots

The viability of two pumpkin varieties was assessed using the standard germination test and compared with the bromothymol blue (BTB) indicator method (Table 8). For the variety Pusa Vishwas (2024), which had an actual germination of 100%, the BTB method estimated 93% viability, showing a 7% deviation from the standard germination test. For Pusa Vishwas (2021), which had an actual germination of 70%, the BTB-estimated viability was 72%, with only a

2% deviation. In the case of normal germination (seedling evaluation as per ISTA, 2025), the deviations ranged from 4% to 31%.

ANOVA (Suppl. Table S16) shows the significant variation among varieties for germination, while differences between the two germination assessment methods (total germination and BTB method) were non-significant (F=1.25, *P*=0.3). The high coefficient of determination (R^2= 0.95) indicated strong agreement between the methods, and the coefficient of variation (CV=4.3%) suggested low variability. In contrast, ANOVA (Suppl. Table S17) showed significant differences between normal germination (standard test) and viability estimated by the BTB method (F=10.44, *P*=0.01), with an R^2=0.88 and a higher CV of 12.5%. Indicating that BTB method can clearly determine the total germination in pumpkin and not suitable for estimating the normal germination as per ISTA, 2025.

Table 8. Comparison of germination percentage between standard germination test and estimation using BTB method in pumpkin varieties.

Varieties	Total G (%)	Percent germination	Estimated viability (%)[#]	Deviation from total G (%)	Deviation from (Percent germination)
Pusa Vishwas (2024)	100 (90)*	89 (70.93)	93 (74.66)	7	4
Pusa Vishwas (2021)	70 (56.58)	41 (40.01)	72 (58.05)	2	31

*Values in the parenthesis are arcsine-transformed values. Total G (%) includes all germinated seed. Percent germination is based on ISTA, 2025 criteria for seedling evaluation. [#]Viability estimated by BTB method.

Significant differences were observed in vigour indices of two seed lots of variety Pusa Vishwas. Pusa Vishwas (2024) had highest vigour index-I (1321.33) and index-II (26.81), while and Pusa Vishwas (2021) showed lowest vigour index I (825.61) and vigour index II (15.22) (Fig. 34 a & b).

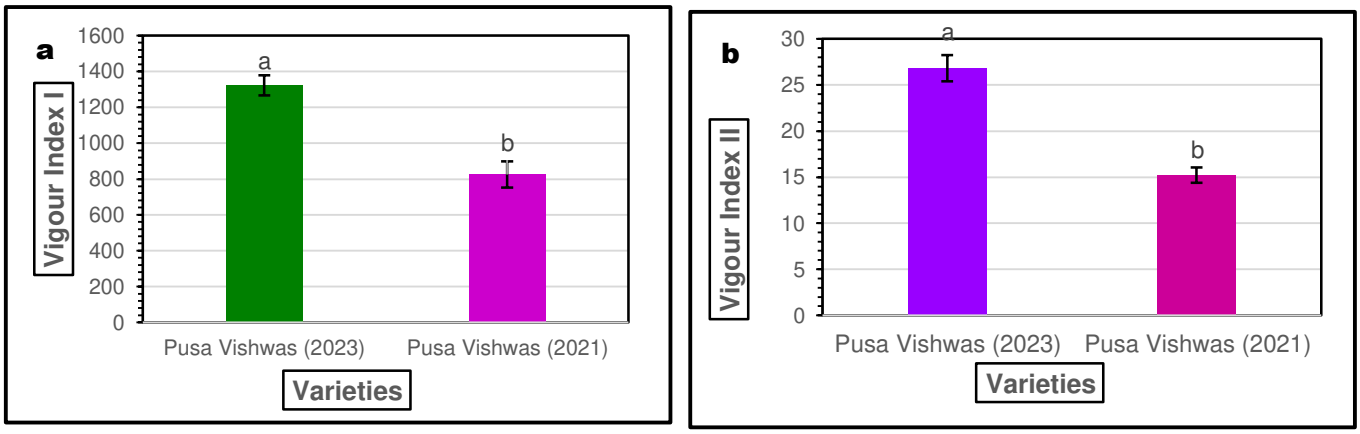


Fig. 34 Vigour indices of two pumpkin seed lots of the variety Pusa Vishwas: (a) vigour index-I and (b) vigour index-II. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences between the 2023 and 2021 seed lots ($P < 0.05$).

Optical density of BTB indicator in pumpkin seed lots varying in germination and vigour

The viability of the two pumpkin varieties estimated by the BTB method was validated through spectrophotometric analysis, wherein the optical density (OD) at 450 nm of the BTB indicator solution was measured after incubation. The OD value for variety Pusa Vishwas (2024) having the germination of 100% was 2.66**,** while that for variety Pusa Vishwas (2021) having the germination percentage of 70% was 1.94 (Fig. 35).

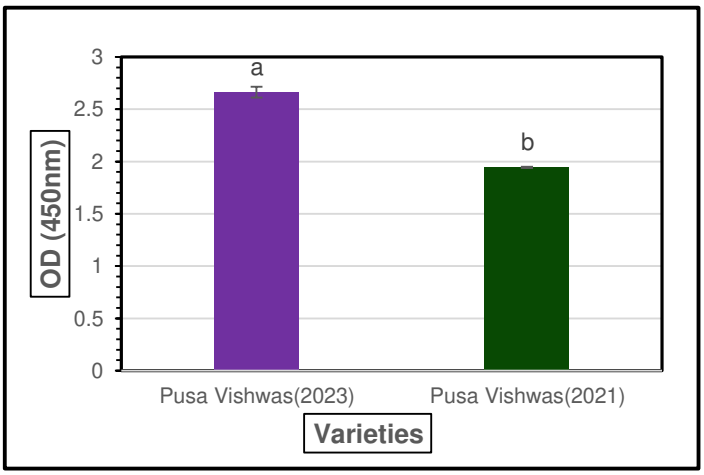


Fig. 35 Optical density (OD) values of bromothymol blue solution after incubation with seeds of two pumpkin seed lots (variety Pusa Vishwas). Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard error. Different letters denote significant differences between the 2023 and 2021 seed lots of variety Pusa Vishwas ($P < 0.05$).

Discussion

Seed quality is an important quality parameter which determines the field establishment and farm productivity. Seed quality must ensure good physical purity, uniform plant stand, and synchronized growth and maturity. Good quality seeds must withstand adverse conditions, respond effectively to inputs, produce vigorous and healthy seedlings with better resistance to pests and diseases, and ultimately lead to increase the productivity, food security and increase the farmers income [41. Several factors influence the initial quality of the seed starting from crop management factors such as nutrient and water availability during growth, climatic conditions during seed development and maturation, and the operation followed in harvesting, threshing, processing and drying [42].

No matter how the seeds are stored, seed ageing or seed deterioration is an inevitable process [5]. With the progressive loss of seed quality, vigour, and viability the period due to natural ageing, leading to reduced germination, weak seedlings, and eventual seed death [43–45]. Seed aging leads to impairment of several cellular functions including seed respiration [17].

Variation in CO_2 evolution in different crops

Respiration is a one of the earliest biological or metabolic processes that occur in the dry seeds upon imbibition, resulting in the consumption of oxygen and the generation of carbon dioxide [24]. The measurement of metabolic activity in seeds has a long-standing history [46], where measurement of respiration is one of the important parameters of seed metabolism [47–49]. Seed respiration is a vital physiological process that plays a key role in seed function. Studying seed respiration is therefore essential for understanding the physiological and biochemical mechanisms underlying seed vigour, metabolism, and germination [21, 50, 51). As the seed ages, the decrease in the rate of aerobic respiration, while increase in the anaerobic respiration [17]. We conducted the study to assess the respiration rate in seven different crops (maize, wheat, green gram, soybean, broccoli, pumpkin, and cucumber) by measuring the CO_2 evolution with the help of infrared CO_2 sensor. Our results suggest that there is a significant difference in the CO_2 evolution during 4 h imbibition period, where the respiration rate in green gram was highest (1876 ppm $seed^{-1}$). The lowest respiration rate has been observed in broccoli seed (487 ppm $seed^{-1}$) (Figure 6).

The CO_2 evolution takes place in the mitochondria of the active living cell [52, 53]. In seeds not all tissues are live, the live tissues include embryo, cotyledon and aleurone layer. The starch and other reserve portion of the seeds comprises of the non-living tissue [54, 55]. In general, when seeds of similar size or weight are compared between monocots and dicots, dicot seeds have a higher proportion of living tissue. This is because a larger portion of dicot seeds—particularly leguminous seeds—consists of cotyledons, which are living tissues [56, 57]. The difference between the rate of CO_2 evolution between the green gram and broccoli can be attributed to the seed mass difference (Suppl. Table S18). But when compared to CO_2 evolution in green gram and soybean, both being dicot, though soybean had higher seed weight (150.7 mg $seed^{-1}$) compared to green gram (43.1 mg $seed^{-1}$) the CO_2 evolution was lower (824 ppm

seed^{-1}). This could be attributed to the difference in the water uptake rate between the two, Luan *et al*., (2017) [58] reported that green gram seeds had highest rate of water uptake compared to soybean. Enhanced water uptake may have contributed to faster metabolic activity though further studies are required. Valle *et al.* (2021) [24] reported that the relatively higher respiration rate observed in corn seeds with the increase in seed moisture, it was attributed to increase in metabolic activity. Jian *et al.* (2014) [59] reported that soybean exhibited higher respiration rates compared to wheat and canola, however, the soybean seeds had a moisture content of 23%, corresponding to a water activity of 0.90. Lacey *et al.* (1994) [60] also found a lower respiration in oilseeds (rapeseed and linseed) than in wheat.

Seed size is an important aspect to consider when evaluating respiration [51]. Apart from seed size it is important to note that comparison to cereals, the pulses have more living tissues (cotyledons), which contribute to the CO_2 evolution, whereas in case of cereals like maize, the size of embryo is small and major portion of seed is occupied by endosperm, which is a non-living tissue. Similarly, our results show that, the respiration rate was relatively higher in the legumes seeds where the seed size of legume was comparatively more than the cereals seeds [61]. The Valle *et al.* (2021) [24] compared the seed respiration of cereals and oilseeds. The respiration rate in maize was higher due to more seed size (Suppl. Table S18). For example, 1000 kernels weight of corn is around 380 g, while that of wheat is 30–45 g, soybeans 150–220 g and rice 27 g [62–65].

Bradford *et al.* (2013) [21] pointed out that the size of the embryo, compared to other parts of the seed, can influence the variation in respiration rate. the embryo of the corn seed weights about 8 mg [66], representing 3–4% of the whole seed. In contrast, wheat embryo weighed about 0.35-0.50 mg, which was more than 10 times smaller representing only 0.9% of the seed [67]. So that, in cereal grains, the embryo makes up just 2–4% of the dry weight, while the

endosperm forms the main structural part, accounting for about 60–85% of the total weight [68].

In the present study, although pumpkin and cucumber seeds have larger living tissues (cotyledons) compared to the other studied crops, which could contribute to higher CO_2 evolution, they exhibited lower respiration rates within the 4-hour observation period—539 ppm seed^{-1} for pumpkin and 525 ppm seed^{-1} for cucumber (Fig. 6). This lower respiration might be attributed to their thicker seed coats, which could have limited water uptake during the imbibition period [69]. However, since the water status of the cotyledon or embryo was not measured in this study, further investigation is needed to confirm this hypothesis.

Variation in respiration rate in seed lots with varying germination percentage

To examine whether respiration varies with seed germination, a study was conducted in which the respiration rate (CO_2 evolution) was measured in different seed lots. The results revealed significant variation among seed lots of maize and soybean corresponding to differences in their germination percentages (Figures 8 and 10). In maize, respiration rates were assessed across three varieties with germination ranging from 100% to 48%. The results indicated that CO_2 evolution over time was significantly related to with percent germination ($P < 0.001$) (Suppl. Table S2). The variety AGRA 2024, which had the highest germination (100%), showed the greatest CO_2 evolution (1053 ppm seed^{-1}), while the variety IML-242-I, with 48% germination, recorded the lowest CO_2 evolution (Figures 8 and 9). Similar trends were observed in soybean seed lots, where germination ranged from 98% to 48%. Here too, CO_2 evolution over time was significantly related to percent germination ($P < 0.0001$) (Suppl. Table S3). The variety SL 955, with 98% germination, exhibited the highest CO_2 evolution (1806 ppm seed^{-1}), whereas the variety JS 0560, with the lowest germination (48%), showed the lowest CO_2 evolution (973 ppm seed^{-1}) (Figure 10).

Dalziell, & Tomlinson (2017) [70] measured the CO_2 production ($\dot{V}CO_2$) using flow through respirometry in seeds lots of 12 species of angiosperms differing in viability, the results proved that there was a significant relationship viability and CO_2 production with r^2 value of 0.92 in maize, 0.65 in onion, 0.88 in pea, 0.59 in tomato. Jiang *et al*. (2018) [71] reported that the respiration rate is a reliable indicator of seed viability in Chinese cabbage with strong correlation found between respiration activity of seeds and germination rates. Yulinda *et al.* (2000) [72] evaluated seed viability in corn, mung bean, and soybean using a titration-based method to measure respiration rates. The results showed that CO_2 measurement could effectively differentiate seed viability in mung bean, but not in soybean and maize. Widajati *et al.* (2023) [73] developed a CO_2 measuring device capable of real-time monitoring using a CO_2 sensor integrated with an IoT system. The experiment included six levels of soybean seed viability (94.8%, 92.4%, 90.4%, 88.4%, 66.0%, and 40.8%, as determined by standard germination tests). Findings of the present study indicated that CO_2 measurements could effectively utilize to distinguish seed lots with different levels of viability.

Loss of seed viability due to aging

During the process of seeds aging, the ability of the seed to germinate, field establishment will declines and the aged seeds pass through various physiological, biochemical, and molecular changes, which negatively affects crop yields, economic returns, and the preservation of genetic resources—leading to substantial commercial and genetic losses [74–77] Reactive oxygen species (ROS) are highly reactive and toxic, capable of destroying cell membranes, nucleic acids, proteins, carbohydrates, and lipids [9]. The production and accumulation of ROS depend on the metabolic and physiological state of the seed [10, 11]. The ROS species such as hydroxyl radical, hydrogen peroxide, superoxide, singlet oxygen, which have the capacity to cause the oxidative damage, can weaken the integrity of the cell membrane in seeds during aging. Lipid peroxidation, which is brought on by ROS, can result in membrane lipid

breakdown and a loss of membrane integrity [12]. Consequently, there may be a decrease in seed viability due to the leakage of electrolytes and other biological components from the are highly reactive and toxic, capable of destroying cell membranes, nucleic acids, proteins, carbohydrates, and lipids [9].

In this study, germination ability was assessed in seven different crops namely maize, wheat, soybean, green gram, broccoli, cucumber, and pumpkin, where the results showed that there was significant difference in the seed germination in seed lots of different crops. As these seeds lot or varieties used in this study has been harvested in different years and stored, so there was significant reduction in the viability. Garoma *et al.,* (2017) [78] indicated that prolonged seed storage led to delayed germination onset, reduced germination rate and index, slower seedling emergence, and lower seedling weight in maize seed lots, where freshly harvested seeds of A-7033 showed highest germination index than the seeds stored and harvested in 2012. Similarly, in this study, AGRA 2024 seed lot was having highest germination 100%, which harvested in 2024, and lowest germination was by the PML-92 which was harvested in 2016 (Table 2). Study conducted by Postic *et al.,* 2023 [79] in three different hybrids of cucumber showed that significant reduction in the germination ability of seed stored for five years. Similarly, study conducted by Alhamdan *et al.,* 2011 [80] in tomato, onion, carrot, cucumber seed stored in different storage temperature condition, results obtained is significant reduction in the germination of the seed in high storage temperature condition. Loss of viability curves during storage at 75% RH and 35˚C in soybean seeds [81].

Viability assessment by using the BTB method

In this study, we aimed to develop a rapid method for assessing seed viability of seed in different crop crops —maize, wheat, green gram, soybean, broccoli, pumpkin, and cucumber by using the BTB indicator. The viability estimates obtained using the BTB method closely

matched the total viability (Tables 2–8). In the present study, the variation in viability estimated visually by BTB method remained within 0–9% of the total germination percentage (ISTA, 2025). The principle underlying this method is that viable and vigorous seeds release more CO_2 than non-viable or low-vigour seeds [21, 27, 28]. Several studies have related the respiration as a reliable indicator for viability and vigour assessment. Powell 2017 [51], and Bradford *et al.,* 2013 [21] measured the oxygen consumption of individual seeds in a closed chamber as a reliable indicator for seed viability and vigour estimation.

In this study, after the visual assessment of individual seed viability by using the BTB method, was also validated spectrophotometric analysis by measuring the optical density of the solution at 450nm after the completion of incubation period. The optical density of seed lot having the high germination has observed highest than the seed lot having low germination. In this optical density has measured in each seed lots of seven different crops studied, for example the OD value of variety AGRA 2024 having the highest germination of 100% was 2.48 and lowest value of 1.033 in variety PML 92 having the 0% germination (Figure 12). Similar results have obtained in all the crop studied which was depicted in the result section of this study.

Linear regression between the optical density (OD) values (measured at 450 nm) of bromothymol blue solution after incubation reveal a significant positive with r^2 ranging from 0.87 to 0.9 (Figures 14, 18, 22, 28, 32). These findings further strengthen the relationship between seed respiration and viability.

Similarly, Min *et al.* (2011) [82] have developed seed leached based rapid assess the viability of single seed by using resazurin in radish seeds, in which due to seed deterioration of seed membrane in aged seed lots due to ROS induced damage to cellular constituents including mitochondria has been effectively used to find germination of seed lots colorimetrically. The OD of radish seed at 600 nm was highest in vigorous seed.

Differentiation of seed lots with different vigour level

Seed vigour is one of the important attributes of seed quality, and seeds with same per cent germination but different vigour levels can perform differently in the field [83, 84]. Seed vigour is commonly estimated using techniques such as vigour indices [40], field emergence [85], and radicle emergence [86] etc. However, these methods are not only laborious but also time-consuming. One of the aims of the study was also to device a reliable quick seed vigour test capable of distinguishing the seed lot of a crop into different vigour, within few hours, compared with several days required by conventional vigour tests.

In the present study after assessing the seeds using the BTB method, the OD values of the indicator solution were measured spectrophotometrically at 450 nm and compared with the vigour indices [40]. The OD values obtained after the incubation period showed a strong positive correlation with seed vigour. A significant positive relationship was observed between vigour index I and OD values, with r^2 ranging from 0.78 to 0.98 (Figures 15a, 19a, 23a, 29a and 33a), and between vigour index II and OD values, with r^2 ranging from 0.87 to 1.00 (Figures 15b, 19b, 23b, 29b and 33b). These findings confirm that OD measurement can serve as a reliable indicator for estimating seed vigour.

Several previous studies using advanced sensing technologies have demonstrated that seed respiration is a reliable indicator for estimating seed vigour [27, 50, 51]. Powell (2017) [51] evaluated the respiration of individual seeds using the Q2 sensor to assess the viability and vigour of seed lots in various crops. Similarly, in vegetable crops, the ASTEC values generated by the Q2 sensor were used to determine seed viability and vigour [21].

Conclusion

This study introduces the rapid colorimetric seed viability and vigour test based on seed respiration. By measuring the CO_2 released during early imbibition, the method directly links

respiratory activity to the seed's physiological status. Variations in CO_2 evolution among different species and seed lots corresponded to differences seed vigour and germination potential. The strong positive correlation between the bromothymol blue OD values and standard vigour parameters confirms respiration as a sensitive and indicator of seed quality assessment. Assay mainly depends on the principle of seed respiration behaviour, which varied from crop to crop, respiration rate was higher in dicot seeds than the monocot seeds. The assay demonstrated rapid assessment across seven crops, but its performance may be influenced by factor such as seed size, metabolic status, water uptake behaviour and incubation condition, which require standardisation for broader application. The rapid colorimetric assay not utilised in small seeds crops like tomato, onion, mustard etc, which are low respiring seed. The vigour of seed depends on the several environmental and storage factor, so there is a need for standardisation in various stress condition. Overall, this respiration-based approach complements the conventional methods by enabling early and rapid assessment of seed quality, and it also offers a useful experimental tool for studies on seed metabolism, priming response, and stress induced changes in respiration dynamics. testing and deepens scientific insight into respiratory dynamics of seed.

Abbreviations

BTB Bromothymol blue

ISTA International Seed Testing Association

CO_2 Carbon dioxide

OD Optical density

nm Nano meter

ppm Parts per million

ROS Reactive oxygen species

ANOVA Analysis of variance

VCO_2 Volume of carbon dioxide produced

pH Potential of hydrogen

IDE Integrated development environment

TNS Total normal seedlings

GMAX Total germination

NaOH Sodium hydroxide

HCO_3^- Bicarbonate

CRD Completely randomized design

SAS Statistical analysis system

AUC Area under curve

Total G (%) Total germinated seeds

Supplementary Information (brief legends)

Arduino Code S1 Arduino code for MH Z19 CO_2 sensor

Table S1 Repeated Measures ANOVA Table for CO_2 Evolution Across Different Crops Over Time

Table S2 Repeated Measures ANOVA Table for CO_2 Evolution Across Maize Varieties Over Time.

Table S3 Repeated Measures ANOVA Table for CO_2 Evolution Across Soybean Varieties Over Time.

Table S4 ANOVA for the effect of varieties, replications, and methods (for total Germination) in maize.

Table S5 ANOVA for the effect of varieties, replications, and methods (for normal seedlings) in maize.

Table S6 ANOVA for the effect of varieties, replications, and methods (for total Germination) in wheat.

Table S7: ANOVA for the effect of varieties, replications, and methods (for normal seedlings) in wheat.

Table S8 ANOVA for the effect of varieties, replications, and methods (for total Germination) in soybean.

Table S9 ANOVA for the effect of varieties, replications, and methods (for normal seedlings) in soybean.

Table S10 ANOVA for the effect of varieties, replications, and methods (for total Germination) in green gram.

Table S11 ANOVA for the effect of varieties, replications, and methods (for normal seedlings) in green gram.

Table S12 ANOVA for the effect of varieties, replications, and methods (for total Germination) cucumber.

Table S13 ANOVA for the effect of varieties, replications, and methods (for normal seedlings) in cucumber.

Table S14 ANOVA for the effect of varieties, replications, and methods (for total Germination) broccoli.

Table S15 ANOVA for the effect of varieties, replications, and methods (for normal seedlings) in broccoli.

Table S16 ANOVA for the effect of varieties, replications, and methods (for total Germination) pumpkin.

Table S17 ANOVA for the effect of varieties, replications, and methods (for normal seedlings) in pumpkin.

Table S18 100 seed weight of seven crops

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Author contributions

VRY, MNE and RK conceived and designed the experiments. MNE, VRY and J performed the experiments. DS analyzed the data. DV, GPM, BST, GSJ, CS, GM were involved in technical advice and discussion. MN and VRY and wrote the manuscript. DV and VHP reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability

All the data supporting the findings of this study are available within the paper and its supplementary information.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests

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