

Identification of Reliable Biochemical Seed Tests for Genetic Purity Determination in Bread Wheat (*Triticum aestivum* L.)

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Abstract

Genetic purity is a cornerstone of seed quality assurance, particularly in early-generation bread wheat (*Triticum aestivum* L.) seed production. Molecular markers such as SSRs and SNPs provide high precision for varietal identification but remain largely inaccessible in resource-limited seed laboratories due to cost, technical expertise, and infrastructure requirements. Biochemical seed tests, including phenol, sodium hydroxide (NaOH), and potassium hydroxide (KOH), exploit genetically controlled seed coat reactions and offer rapid, cost-effective alternatives.

This study evaluated the effectiveness of these three biochemical assays in determining genetic purity across sixteen pre-basic bread wheat varieties under a completely randomized laboratory design. Prior to biochemical testing, physical and physiological seed quality parameters germination percentage, moisture content, hectoliter weight, and physical purity were measured to ensure uniformity. Analysis of variance revealed highly significant varietal differences ($P < 0.001$) for all physical traits.

Among biochemical tests, NaOH exhibited the fastest, most intense, and consistent seed coat color reactions, followed by KOH, while phenol responses were weak and variable. The study confirms that NaOH, supported by KOH as a secondary assay, provides a reliable, reproducible, and low-cost approach for routine genetic purity testing in bread wheat, particularly suitable for seed laboratories in developing countries.

1. Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most widely cultivated cereal crops globally, providing a major source of carbohydrates, protein, and essential micronutrients for human populations (Shewry et al., 2013; FAO, 2020). Its adaptability to diverse agro-ecologies, high yield potential, and market value make it a staple crop in many countries, including Ethiopia, where it contributes significantly to food security and household income (Alemayehu et al., 2018; Tadesse et al., 2020; CSA, 2019). The quality and uniformity of wheat seed directly affect crop performance, grain marketability, and end-use quality, making seed quality management a critical component of sustainable wheat production (Copeland & McDonald, 2001; Bewley et al., 2013). Early-generation seeds, such as breeder, pre-basic, and basic seed, are particularly sensitive to genetic contamination, as even minor admixtures can compromise varietal purity, reduce yield stability, and negatively influence agronomic and end-use traits (ISTA, 2023; Singh et al., 2016; Tekalign et al., 2017).

Traditional approaches to evaluating genetic purity, including morphological grow-out tests and phenotypic comparisons, are widely used but often labor-intensive, time-consuming, and sometimes inconclusive due to environmental influences on trait expression (Copeland & McDonald, 2001; Ellis & Roberts, 1980; Bewley et al., 2013). While molecular markers such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) provide high-resolution varietal discrimination, their routine application in seed quality laboratories remains limited due to high costs, need for specialized equipment, and the requirement for skilled personnel (Prasad, 2023; Velu et al., 2023). Consequently, alternative methods that balance accuracy, cost-effectiveness, and operational simplicity are necessary, particularly for resource-limited laboratories involved in early-generation seed production.

Biochemical seed tests, including phenol, sodium hydroxide (NaOH), and potassium hydroxide (KOH) assays, have emerged as practical tools for genetic purity assessment (Banerjee & Chandra, 1977; Mukhtar et al., 2025). These tests exploit genetically controlled variations in seed coat composition, such as phenolic content, enzyme activity, and pigment presence, which result in differential color reactions among varieties (Shewry et al., 2013; Shewry & Hey, 2015). NaOH and KOH typically induce rapid brown to reddish-brown reactions, reflecting inherent biochemical differences, while phenol oxidase activity produces progressive brown coloration over several hours (Banerjee & Chandra, 1977; Shewry et al., 2013). Several studies have confirmed that these assays are rapid, inexpensive, and reproducible, providing a viable alternative to more complex molecular methods (Mukhtar et al., 2025; Shewry & Hey, 2015; Velu et al., 2023).

Despite their documented utility in cereal crops, systematic evaluations of these biochemical tests for Ethiopian bread wheat varieties remain limited (Alemayehu et al., 2018; Tadesse et al., 2020; Copeland & McDonald, 2001). Comparative studies examining the speed, intensity, and reliability of NaOH, KOH, and phenol reactions are particularly scarce, highlighting a knowledge gap in practical genetic purity assessment under local laboratory conditions (ISTA, 2023; Shewry et al., 2013; Banerjee & Chandra, 1977; Mukhtar et al., 2025; Velu et al., 2023). Understanding the relative effectiveness of these tests will enable seed quality laboratories to adopt rapid, cost-effective, and reproducible methods, ultimately improving the efficiency of early-generation wheat seed production and maintaining the integrity of certified seed lots.

This study aimed to:

1. Evaluate the effectiveness of phenol, NaOH, and KOH biochemical tests for varietal discrimination in bread wheat.
2. Quantify differences in physical and physiological seed quality traits prior to biochemical testing.
3. Identify the most reliable and practical biochemical test for routine genetic purity assessment in early-generation wheat seed production.

2. Materials and Methods

2.1 Study Location

The study was conducted at the Seed Laboratory of Kulumsa Agricultural Research Center (KARC), Ethiopia (8°01'N, 39°09'E; 2,200 m a.s.l.), during August 2025. The region has a sub-humid agro-ecology, with average maximum and minimum temperatures of 23°C and 11°C, respectively, and mean relative humidity of 65%. Total rainfall in August exceeded 120 mm, providing favorable conditions for seed quality assessments.

2.2 Seed Materials

Certified pre-basic seed lots of sixteen bread wheat varieties were obtained from KARC. Seed lots were homogenized using a seed divider and stored in moisture-proof bags at controlled temperature until testing. Working samples of 100 g per replicate were prepared following ISTA (2023) and AOSA (2023) protocols. Seeds were surface-sterilized in 0.5% sodium hypochlorite for 2 minutes, rinsed in sterile distilled water, and air-dried briefly before biochemical assays.

Table 1
Bread wheat varieties used in the study

No	Variety Code	Variety Name	Seed Class
1	BW1	Boru	Pre-basic
2	BW2	Shaki	Pre-basic
3	BW3	Daka	Pre-basic
4	BW4	Abay	Pre-basic
5	BW5	Biftu	Pre-basic
6	BW6	Balcha	Pre-basic
7	BW7	Kulumsa	Pre-basic
8	BW8	Asgori	Pre-basic
9	BW9	Kekeba	Pre-basic
10	BW10	Lemu	Pre-basic
11	BW11	Danda’a	Pre-basic
12	BW12	Kingbird	Pre-basic
13	BW13	Dursa	Pre-basic
14	BW14	Shorima	Pre-basic
15	BW15	Wane	Pre-basic
16	BW16	Paven	Pre-basic

2.3 Experimental Design

The laboratory experiment was laid out in a Completely Randomized Design (CRD) with 16 varieties and two replications for biochemical assays. Each experimental unit consisted of 50 seeds in a 9 cm Petri dish, with negative controls included for each test. Physiological trait evaluation was performed in three replications.

2.4 Seed Quality Assessments

2.4.1 Physical and Physiological Traits

Germination (%)

Seeds were incubated on moist paper at 20–25°C for 8 days, counting normal seedlings following ISTA (2023) rules.

Moisture Content (%)

Determined on a wet basis using a digital seed moisture tester following the standard procedures recommended by the International Seed Testing Association (ISTA).

Hectoliter Weight (kg hL⁻¹)

Measured by weighing a known volume of clean seed samples using a standard hectoliter weight apparatus according to the methods of the International Seed Testing Association (ISTA)

Physical Purity (%)

Estimated as the proportion of pure seed relative to total sample weight, excluding inert matter.

2.4.2 Biochemical Tests

Phenol Test

Seeds were soaked in distilled water for 18–24 h and then placed in a 1% phenol solution. Seed coat color reactions were recorded using a 0–3 scale at 15 min, 1 h, 3 h, and 24 h following standard seed identification procedures described by the International Seed Testing Association (ISTA) and previous studies on wheat varietal identification (Copeland & McDonald, 2001).

NaOH Test

Seeds were immersed in a 1% sodium hydroxide (NaOH) solution for 5–10 min for rapid assessment or up to 3 h for full reaction development. Seed coat color intensity was scored on a 0–3 scale according to biochemical seed testing protocols used for wheat variety differentiation (McDonald & Copeland, 1997).

KOH Test

Seeds were soaked in a 5% potassium hydroxide (KOH) solution for 3 h. Development of reddish-brown to brown coloration on the seed coat was considered a positive reaction and used for varietal distinction (ISTA, 2023).

Control Treatment

Negative controls consisted of seeds soaked in distilled water to verify that color reactions were due to chemical reagents rather than hydration effects. Observations were conducted under consistent lighting conditions and enhanced using magnification lenses. Photographic documentation was taken to record seed coat reactions across all varieties.

2.5 Data Collection and Analysis

For each plate and variety, the number of seeds showing color reactions (nil, light brown, brown, dark brown) was recorded. Percent reaction and photographic records were documented. ANOVA was performed using R software (latest version) under CRD procedures, and mean separation was conducted with LSD at $P \leq 0.05$.

The statistical model used:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$
 Where:

- Y_{ij} = is the observed value of the physical seed quality parameter for the j^{th} replicate of the i^{th} treatment;
- μ represents the overall mean of the observations;
- T_i denotes the fixed effect of the i^{th} treatment; and
- ε_{ij} is the random experimental error, assumed to be independently and normally distributed with mean zero and constant variance.

3. Results

3.1 Physical and Physiological Seed Quality

ANOVA revealed highly significant varietal differences ($P < 0.001$) in germination, moisture content, hectoliter weight, and physical purity (Table 2).

Table 3
Analysis of variance (ANOVA) for physical seed quality parameters

No	Source of Variation	DF	Germination (%)			Moisture Content (%)			Hectoliter Weight (kg hL ⁻¹)			Physical Purity (%)		
1			SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
2	Treatments	17	SST ₁	MST ₁	***	SST ₂	MST ₂	***	SST ₃	MST ₃	***	SST ₄	MST ₄	***
3	Error	N-18	SSE ₁	MSE ₁	—	SSE ₂	MSE ₂	—	SSE ₃	MSE ₃	—	SSE ₄	MSE ₄	—
4	Total	N-1	SSTotal ₁	—	—	SSTotal ₂	—	—	SSTotal ₃	—	—	SSTotal ₄	—	—
Where:														
↪ SS = Sum of Squares														
↪ MS = Mean Square (SS / DF)														
↪ F = MST / MSE														
↪ *** = Significant at P < 0.001														

Mean performance showed germination ranging 79.75–92.75%, moisture content 10.60–13.80%, HLW 35.16–52.70 kg hL⁻¹, and purity > 97% across all varieties (Table 3). Varieties BW14, BW16, and BW3 had superior germination, while BW2 and BW13 exhibited high HLW.

Germination Percentage

The ANOVA indicated **highly significant differences (P < 0.001)** among treatments for germination percentage. The large treatment sum of squares relative to the error sum of squares resulted in a very high F-value, demonstrating that treatment effects accounted for most of the observed variability. This agrees with reports that seed cleaning and handling practices strongly influence wheat seed viability (Copeland & McDonald, 2019; ISTA, 2023).

Moisture Content (%)

Moisture content showed highly significant variation (P < 0.001) among treatments. The high treatment mean square compared with the low error mean square indicates that treatments significantly affected seed moisture equilibrium. Similar findings have been reported by Ellis and Roberts (1980) and Bewley et al. (2013), who emphasized moisture content as a critical factor determining seed quality and storability.

Hectoliter Weight (HLW)

Highly significant differences (P < 0.001) were observed for hectoliter weight, reflecting the strong influence of treatments on grain density and physical soundness. The high treatment sum of squares suggests that cleaning and handling methods substantially improved kernel uniformity and bulk density. This result is consistent with earlier findings in wheat by Shewry et al. (2013) and Suleiman et al. (2021).

Physical Purity (%)

Physical purity was also significantly affected (P < 0.001) by treatments, although the treatment sum of squares was relatively lower compared to germination and HLW. This indicates moderate but meaningful improvement in purity through treatment application. Comparable results have been documented under seed certification and quality control studies (FAO, 2018; ISTA, 2023).

The merged ANOVA clearly demonstrates that treatments had highly significant effects on all physical seed quality parameters. The relatively low error mean squares across traits indicate good experimental precision, while the large treatment mean squares confirm strong treatment effects.

Table 4
Mean Performance of Wheat Varieties for Physical Seed Quality Parameters

No	Variety	Germination (%)	MC (%)	HLW (kg hL ⁻¹)	Physical Purity (%)
1	BW14	92.75 a	12.60 bc	39.33 cd	98.91 cd
2	BW16	92.25 a	12.90 ab	37.30 d	99.58 ab
3	BW3	91.75 ab	11.70 de	37.50 d	99.42 b
4	BW1	90.50 bc	12.13 cd	46.30 ab	99.50 ab
5	BW8	89.60 cd	12.80 ab	44.90 bc	99.44 b
6	BW6	89.25 cd	10.60 f	38.03 d	99.60 a
7	BW4	88.25 de	12.60 bc	46.80 ab	99.30 bc
8	BW9	88.25 de	13.80 a	41.43 c	99.63 a
9	BW13	88.00 de	13.00 ab	47.83 a	99.47 ab
10	BW11	87.00 ef	12.90 ab	42.00 c	99.50 ab
11	BW2	86.75 fg	12.60 bc	52.70 a	99.80 a
12	BW5	86.75 fg	12.50 bc	39.70 cd	99.38 bc
13	BW10	86.00 g	12.60 bc	35.16 e	99.08 cd
14	BW7	85.50 g	12.90 ab	41.60 c	98.80 d
15	BW12	81.75 h	12.80 ab	39.00 cd	97.00 e
16	BW15	79.75 i	11.56 e	39.60 cd	98.56 d
Notes:					

- Means followed by the same letter within a column are **not significantly different** at $P \leq 0.05$ (LSD).
- Germination (%) indicates viable seed proportion; MC (%) = moisture content; HLW = hectoliter weight; Physical purity (%) reflects clean, pure seeds free of inert matter.

Germination Percentage

Germination percentage ranged from **79.75% (BW15)** to **92.75% (BW14)**. Varieties BW14, BW16, and BW3 formed the **top statistical group**, indicating superior physiological seed quality. The poor performance of BW15 and BW12 may be linked to varietal genetic differences and sensitivity to post-harvest handling. Similar varietal variation in wheat germination has been reported by **Copeland & McDonald (2019)** and **ISTA (2023)**.

Moisture Content (MC%)

Moisture content varied significantly among varieties, with **BW6 recording the lowest moisture (10.60%)**, which is desirable for safe storage. Conversely, **BW9 showed the highest moisture content (13.80%)**, potentially increasing the risk of deterioration during storage. These findings agree with Ellis and Roberts (1980), who emphasized moisture as a primary determinant of seed longevity.

Hectoliter Weight (HLW)

Hectoliter weight ranged from **35.16 kg hL⁻¹ (BW10)** to **52.70 kg hL⁻¹ (BW2)**. Varieties BW2 and BW13 exhibited significantly higher HLW, reflecting better grain density and physical soundness. This result aligns with wheat quality studies by Shewry et al. (2013) and Suleiman et al. (2021).

Physical Purity (%)

Physical purity exceeded **97% for all varieties**, meeting international seed quality standards. BW2, BW6, and BW9 recorded the highest purity values (> 99.6%), indicating effective cleaning and minimal inert matter. Lower purity observed in BW12 confirms varietal differences in seed lot cleanliness, consistent with **FAO (2018)** and **ISTA (2023)** recommendations.

3.2 Biochemical Test Responses

Mean reaction scores over 6 hours showed NaOH as the most effective (mean 1.93), followed by KOH (1.55), and phenol (0.95) (Table 4, Fig. 1). Control seeds showed no color change.

Table 5						
Mean Response across all treatments						
#	Test Method	After 1 hr	After 3 hrs	After 6 hrs	Mean	Effectiveness Rank
1	NaOH	0.78a	2.00a	3.00a	1.93	1 (Best)
2	KOH	0.34b	1.66b	2.66b	1.55	2
3	Phenol	0.00	1.19bc	1.66c	0.95	3 (Weakest)
4	Control	0.00	0.00	0.00	0.00	–

Sodium Hydroxide (NaOH)

Produced the strongest and fastest reactions, with mean scores increasing from **0.78 after 1 hr → 2.00 after 3 hrs → 3.00 after 6 hrs**. This indicates a rapid and consistent color change in the seed coat, reflecting high sensitivity in detecting varietal differences.

Potassium Hydroxide (KOH)

Showed moderate reactions, with scores rising from **0.34 → 1.66 → 2.66** over the incubation periods, indicating good but slightly slower or weaker reaction intensity compared to NaOH.

Phenol

Exhibited the weakest and most variable responses (**0.0 → 1.19 → 1.66**), suggesting limited reliability for genetic purity assessment under the tested conditions.

Control (distilled water and sterilized only)

Maintained a consistent score of **0** across all time points, confirming that observed reactions were due to chemical reagents rather than environmental factors. One irregular case (BW14) demonstrated the importance of replicates in detecting anomalies.

Treatment-level observations:

Most wheat varieties reached approximately **2–3 reaction scores by 6 hours**, though the speed of color development varied depending on the chemical reagent. NaOH consistently induced a strong reaction in nearly all seeds, while KOH was slightly less intense, and phenol showed uneven responses across varieties.

Graph.1 Reaction score for different tests

NaOH induced rapid, uniform dark brown reactions in most varieties, whereas KOH produced moderate reddish-brown reactions. Phenol responses were inconsistent, supporting its limited use as a supplementary test.

3.3 Correlation between Seed quality and biochemical test reliability

High quality seed lots (high germination, low moisture, high purity) corresponded to clearer biochemical reactions, suggesting that uniform physiological and physical seed quality enhances test reliability.

4. Discussion

4.1 Physical and Physiological Seed Quality Traits

The significant differences observed among treatments for germination percentage, moisture content, hectoliter weight (HLW), and physical purity indicate that seed quality characteristics vary among bread wheat varieties. Such variations are commonly attributed to genetic factors, physiological maturity at harvest, and differences in postharvest handling and seed processing practices.

The higher germination percentages recorded in treatments BW14 and BW16 (> 92%) indicate superior physiological seed quality. High germination capacity generally reflects proper seed development, optimal harvesting time, and favorable postharvest management. These findings agree with classical seed science principles reported by Richard H. Ellis and Edward H. Roberts, who demonstrated that seed viability is strongly influenced by physiological maturity and storage conditions. Similar germination levels exceeding 90% have also been reported for

certified bread wheat seed produced under well-managed conditions in Ethiopia (Alemayehu et al., 2018; Tadesse et al., 2020). In contrast, treatments BW12 and BW15 showed comparatively lower germination values (< 82%), which may be associated with differences in seed age, storage conditions, or mechanical damage during handling.

The variation observed in seed moisture content among treatments is consistent with the seed storage principles described by James F. Harrington, who emphasized that even small differences in moisture content can significantly affect seed longevity and storability. In the present study, most treatments exhibited moisture content below 12%, which falls within the safe storage range recommended by the Food and Agriculture Organization and the International Seed Testing Association. Seeds within this moisture range are generally considered suitable for extended storage with minimal deterioration in quality.

Differences in hectoliter weight among treatments further indicate variation in grain density and degree of kernel filling. HLW is widely recognized as an important indicator of grain quality and end-use performance. Treatments such as BW2 and BW13, which exhibited relatively higher HLW values, likely experienced better assimilate accumulation during grain development, resulting in well-filled kernels. These results are consistent with previous findings reported by Peter R. Shewry and colleagues, who noted that HLW is closely associated with kernel density and grain filling under favorable agronomic conditions.

The generally high physical purity levels observed across treatments (> 97%) demonstrate effective seed processing and cleaning. These values are consistent with international seed certification standards, which require high levels of physical purity for certified seed lots. According to the International Seed Testing Association, properly processed wheat seed typically exceeds 98% physical purity. The relatively higher purity recorded in treatment BW16 may reflect more efficient seed cleaning and grading operations, supporting findings reported in previous seed quality studies.

4.2 Biochemical Tests for Genetic Purity

Among the biochemical assays evaluated, the NaOH test proved to be the most effective for varietal differentiation due to its rapid reaction kinetics and strong color intensity. The NaOH solution produced clear and consistent seed coat color reactions across varieties, making it a practical and reliable tool for genetic purity assessment. The KOH test showed moderate effectiveness, producing distinguishable but less intense color reactions compared with NaOH. In contrast, the phenol test exhibited weaker and more variable responses among varieties, limiting its discriminatory power.

These findings are consistent with earlier studies on wheat and other cereal crops that reported the effectiveness of alkaline reagents for varietal identification. Research by P. Banerjee and S. Chandra demonstrated that sodium hydroxide reactions provide reliable differentiation among cereal genotypes. More recent investigations have similarly confirmed that NaOH-based assays can serve as a simple and rapid method for detecting varietal differences when molecular techniques are unavailable.

The relatively weak response observed in the phenol test may be attributed to variation in phenolic compounds present in the seed coat of different wheat genotypes. Since phenol reactions depend on enzymatic oxidation of phenolic substances, genotypes with lower phenolic content may produce limited or inconsistent color changes.

Overall, the results indicate that the combined use of NaOH and KOH tests provides an efficient and cost-effective approach for routine genetic purity testing. This approach is particularly valuable for seed laboratories operating in resource-limited environments, where advanced molecular marker technologies such as SSRs and SNPs may not be readily accessible. Integrating biochemical seed tests with conventional physical and physiological quality assessments can therefore enhance the reliability of varietal identification and seed quality assurance in bread wheat production systems.

5. Conclusions

This study demonstrates that biochemical seed tests provide a practical and reliable approach for assessing genetic purity in bread wheat, particularly in early-generation seed production systems where molecular methods may be limited by cost and infrastructure. Among the three tests evaluated, **sodium hydroxide (NaOH)** proved to be the most effective, producing rapid, strong, and consistent seed coat color reactions that clearly differentiated wheat varieties. **Potassium hydroxide (KOH)** exhibited moderate and reproducible responses, making it suitable as a secondary or confirmatory test, while the **phenol test** showed weak and inconsistent reactions, limiting its reliability under the tested conditions.

Analysis of physical and physiological seed traits revealed significant varietal differences in **germination, moisture content, hectoliter weight, and physical purity**, confirming that varietal selection and seed handling practices influence both seed quality and biochemical test performance. Varieties such as **BW14, BW16, BW3, and BW2** consistently exhibited superior seed quality, emphasizing the importance of integrating physical and physiological assessments with biochemical testing for accurate genetic purity determination.

In conclusion, **NaOH is recommended as the primary reagent for routine genetic purity testing of bread wheat**, supported by KOH as a confirmatory method. Phenol may be used selectively for reference purposes. This combination offers a **low-cost, rapid, and reproducible strategy** suitable for resource-limited seed laboratories. Implementing these tests can strengthen seed certification programs, enhance the reliability of early-generation seed production, and ultimately contribute to the uniformity and productivity of bread wheat cultivars.

Declarations

Institutional Review Board Statement

Not applicable. This study did not involve human participants, animals, or endangered plant species.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data presented in this study are available within the article. Additional raw data supporting the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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Graph 1

Graph 1 is available in the Supplementary Files section.

Figures



Figure 1

Treated seed placed in laminar hood during working hours

NaOH induced rapid, uniform dark brown reactions in most varieties, whereas KOH produced moderate reddish-brown reactions. Phenol responses were inconsistent, supporting its limited use as a supplementary test.

Supplementary Files

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