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A spectrum of chemical tests in rice varieties identification and classification

[*]GBN JYOTHI AND [1]S. DAS

Department of Seed Science and Technology,Odisha University of Agriculture and Technology, Bhubaneshwar
[1]Odisha State Seed and Organic Product Certification Agency, Bhubaneshwar, Odisha,

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Abstract

Investigation used a variety of chemical assays to identify and characterize rice varieties. Seed samples of Sixteen rice varieties were used in experimentation which are under cultivation in the state of Odisha. These were released by Odisha University of Agriculture and Technology, Bhubaneswar and National Rice Research Institute, Cuttack. Phenol test clustered sixteen varieties into six distinct groups viz., very pale brown (1 variety), pale brown (5 varieties), brown (5 varieties), deep brown (1 variety), very deep brown in color (3 varieties) and no color change (1 variety). Modified phenol test using $CuSo_4$ solution helped in further sub-division of standard phenol group into four sub-groups viz., pale brown (5 varieties), brown (7 varieties), deep brown (2 varieties), and black color (1 variety). Based on NaOH test varieties grouped into three classes such as no color(1 variety), pale yellow (8 varieties) and yellow (7 varieties).In case of KOH test, varieties grouped into six classes such as no color (1 variety), pale yellow(2 varieties), yellow (3 varieties), pale brown (5 varieties),brown (3 varieties)and deep brown or reddish brown (2 varieties).The genotypes were categorized based on their distinct responses to GA3 in terms of coleoptile length growth as very low response (< 10 %), low response (10-30 %) with eight genotypes and moderate response (>30 %) with eight genotypes. The increase in coleoptile length over control ranged from 14.03 per cent (Jogesh) to 48.87 per cent (Manaswini).

***Keywords*:** Characterization, cultivar purity, GA_3 test, KOH test, modified phenol test, NaOH test and phenol test.

A significant number of crop improvement initiatives in India have resulted many developed varieties. As a result, maintaining the genetic purity and uniqueness of each variety necessitates varietal identification (Rai *et al*., 2019). Genetic purity verification is a key source for sustainable breeding and development of a crop over generations and a key element of seed quality assurance. Vigour, germination, purity, and seed health are variables in seed quality (Reddy *et al.,*2013). So, precise method for determination of genetic purity of cultivar or pre-released variety is required to test for the distinctness of seed and seedling level for the presence or absence of enzymes in seed such as tyrosinase in paddy seed and tannins in sorghum, pigments in seedlings of rye and cotton.

In cultivar purity seeds or plant samplings are analyzed for undesirable varieties by various methods such as morphological analysis, chemical tests, biochemical analysis**,** DNA marker methods, Protein-based isozyme electrophoresis, two-dimensional electrophoresis-isoelectricfocusing, Enzyme-linked immunosorbent assay (ELISA) testing, herbicide, or insect tolerance tests, grow out test etc**.** Though GOT is most reliable method of assessing genetic purity method over above stated but it is not a rapid method of identification which is time-consuming, highly seasonal, quite influenced by various environment factors and individual character expression depends on soil fertility, in addition to it GOT need more labor workforce and infrastructure used (Bora *et al.*, 2008 and Brake Donald, 1995).

Morphological markers are universally accepted for DUS testing, but they are limited for usage of large area, time, labor and affected by environment which result in narrow genetic base. So simple tests like variation of seed coat and seed soaked decant to different biochemicals which is of variety specific and reproducible, had intensified the DUS test for few crops such as Phenol test approved for DUS testing of wheat varieties, KOH bleach approved for DUS testing of Tannic acid in sorghum, Peroxidase activity test for DUS testing of soybean etc. A fast and accurate technique to identify and analyze the purity of seed lot is required to achieve the minimal seed certification standards of seed quality. Probable morphological traits would not be enough to distinguish all the new types and extant one. So, there is a greater need to explore precise alternative methods to characterize cultivars which are laboratory friendly, unaffected by environmental change and labor obsession free.

The current study carried out to ascertain the response of seeds of 16 commercial rice varieties responded to various chemical tests, such as the phenol test, modified phenol (CuSO4) test, potassium hydroxide (KOH) test, and sodium hydroxide (NaOH) test, and to see if these tests could be used for varietal identification and characterization.

Email: jyothigodavari1@gmail.com

MATERIAL S AND METHODS

Seed source

Seed samples of sixteen rice varieties (Upahar, Jogesh, Tejaswini, CRD 202, CRD 500, CRD 505, CRD 508, Jyotirmayee, Prativa, Hiranmayee, Nua Acharmati, Asutosh, Pradeep, Manaswini, Mrunalini and Hasanta) released by Odisha University of Agriculture and Technology, Bhubaneswar and National Rice Research Institute, Cuttack were used for this experimentation. The experiment was conducted at Seed physiology laboratory, Department of Seed Science and Technology, Odisha University of Agriculture and Technology, Bhubaneswar.

Phenol test

In the phenol test, 50 seeds per replication were soaked in distilled water for 16 hours at 25±1°C. The seeds were then placed in petri dishes lined with filter paper, moistened with 4 ml of 1% phenol solution, and maintained at room temperature (28°C) for 24 hours. In such way that ¾ level of seed was immersed in solution and petri plates were closed immediately after adding solution to the seed, so that phenolic vapors did not escape from the petri plate and incubated for 24 hours at 30°C, due to the activity of tyrosinase enzyme change in color of seed coat had diagnosed (Walls,1965).

Modified phenol test

The modified phenol test was carried out using similar methods as the normal phenol test, except instead of soaking the seeds in distilled water, they were immersed in 15 ml of 0.5 per cent CuSO4 solution for 24 hours. Then, they were placed over a moist filter paper with 4ml of 1% phenol solution. Observations and classifications were made based on the seed coat colour reaction into different colour classes such as pale brown, deep brown, and black (Vishwanath *et al*., 2013).

NaOH test

The change in colour of the solution was noticed after four replications of 50 seeds of each cultivar were soaked in 15 ml of 5% NaOH solution and incubated at room temperature for 5 hours. The genotypes were divided into three groups based on the strength of the colour reaction: no colour change, pale yellow, and yellow colour. (Sripunitha and Sivasubramaniam, 2014).

KOH test

Two hundred seeds (50x4) had soaked in 15 ml of 5% KOH solution for 5 hours, after which the cultivars were classified depending on the strength of the colour change as pale yellow, yellow, and reddish-brown / deep brown and no colour change (Sripunitha and Sivasubramaniam, 2014).

GA_3 test

Surface sterilized rice seeds with distil water were placed in a double layer blotter moistened with 25ppm GA_3 solution and left for incubation as per ISTA procedure at 25±100^{0}C, water-soaked blotter without GA_3 solution was used as a control, allowed the incubated seeds to germinate and measured the length of ten random seedlings at the end of the 14th day to note the percent increase in the seedling length. The percentage increase in coleoptile length over control was calculated by using the following formula:

$$\frac{\text{length of } GA_3 \text{ treated coleoptile-length of coleoptile with control}}{\text{length of coleoptile with control}} \times 100$$

The seeds were grouped by the per cent increase of coleoptiles length over the control as follows: a) Very low response:< 10 per cent increase b) Low response: 10-30 per cent increase c) Moderate response:> 30 percent increase.

RESULTS AND DISCUSSION

Chemical tests are rapid, low cost and time effective over conventional method such as grow out test (GOT) which need more land and time to grow a crop and morphological observation should be noted throughout the crop growth period from sowing to harvest (Vishwanath *et al*., 2013). While chemical test based on enzymatic or chemical reaction between seed coat and chemical solution can be noted in few hours in KOH and NaOH tests to few days in Phenol, Modified phenol and GA3 tests.

Standard phenol test

Depending on flavonoid reaction in seed pericarp 16 rice varieties used in the experiment were classified into six classes such as very pale brown, pale brown, brown, deep brown, very deep brown, and no color change (Table 1 and Fig. 1). These findings are consistent with Singh *et al.* (2017) and Rai *et al.* (2019).

Phenol test is dependent on a flavonoid reaction in seed pericarp; color change is due to reaction between vapor of phenol and the glumes which takes place in two reactions, initially phenol hydroxylated aromatic ring form catechol's or quinols, in second reaction catechol's or quinols undergo oxidation to form quinones (Takahashi and Tsunoda,1984; Vishwanath *et al.,* 2013, Kumar *et al.,* 2016; Rai *et al.,* 2019). Phenol test is used as a primary grouping test of genotypes and it should be used initially to group the varieties (Vishwanath *et al*.,

Table 1: Response of rice varieties to phenol tests

Color development	Variety
No color	*Manaswini.*
Very pale brown	*Mrunalini.*
Pale brown	*Asutosh, CRD 202, CRD 508, Upahar, Prativa.*
Brown	*CRD 500, Hasanta, NuaAcharmati, Pradeep, Jogesh.*
Deep brown	*Tejaswini.*
Very deep brown	*CRD 505, Jyotirmayee, Hiranmayee.*

Table 2: Response of rice varieties to modified phenol test

Color development	Variety
Pale brown	*CRD 202, Manaswini, Mrunalini, Prativa, Upahar.*
Brown	*Asutosh, CRD 508, Hasanta, Jogesh, Jyotirmayee, NuaAcharmati, Pradeep.*
Deep brown	*CRD 505, Hiranmayee.*
Black	*CRD 500.*

Table 3: Response of rice varieties to NaOH test.

Color development	Variety
No color	*CRD 505.*
Pale yellow	*CRD 508, Hasanta, Hiranmayee, Jogesh, Manaswini, Mrunalini, Pradeep, Upahar.*
Yellow	*Asutosh, CRD 202, CRD 500, Jyotirmayee, NuaAcharmati, Prativa, Tejaswini.*

Table 4: Response of varieties to KOH test

Color development	Variety
No color	*Jogesh*
Pale yellow	*CRD 202, CRD 500*
Yellow	*Hasanta, Mrunalini, Upahar*
Pale brown	*CRD 505, CRD 508, Hiranmayee, Tejaswini, NuaAcharmati.*
Brown	*Prativa, Pradeep, Manaswini*
Deep brown/reddish brown	*Autosh, Jyotirmayee*

2013) because phenol test is monogenically controlled (Joshi and Banerjee, 1970). The results of the experiment are like with Vishwanath *et al.*(2013) and Anitalakshmi *et al.* (2014) in rice.

Modified phenol test

Addition of CuSo4 to phenol solution in modified phenol test has developed deeper color than phenol test, which helped in further grouping the varieties into four sub-groups as stated below such as pale brown, brown, deep brown, black (Table 2 and Fig. 1). Similar findings are noted by Vishwanath *et al.*(2013) and Singh *et al.* (2017).

Further support was given by a modified phenol test employing CuSo4 solution sub-division of standard phenol group which is also a promising test. The color development in modified phenol test is due to Cu++ ions a co-factor for the hydroxylating enzyme (Jaiswal and Agrawal, 1995). Both phenol and modified phenol tests are suitable for uniform grouping of Indian mustard varieties (Rai *et al.* 2019). Similar observations were made by Gupta *et al.* (2007) in wheat, Vishwanath *et al.* (2013), Sripunitha *et al.* (2014) and Anitalakshmi *et al.* (2014) in rice.

NaOH test

Based on the color developed in NaOH seed-soaked solution 16 rice varieties used in experimentation were grouped into three classes such as no color, pale yellow and yellow (Table 3 and Fig. 1). Similar findings were noted by Vishwanath *et al.*(2013); Singh *et al.*(2017); Rai *et al.*(2019); Raut *et al.*(2019) and Mathad *et al.*(2019).

Because of the chemical contents and genetic makeup of the cultivars, the NaOH and KOH test solutions reacted differently, so variation occured in color used for grouping varieties. The variation in color might be due to difference in chemical inherent, and secondary

Table 5: Identification and clustering of sixteen rice varieties based upon the coleoptile growth response to GA_3 test

Variety	GA_3 (cm)	Control (cm)	Percentage increased over control	Groups
Manaswini	39.6	26.6	48.87	Moderate
Hiranmayee	38.8	28.4	36.62	Moderate
CRD 202	36.24	28.56	26.89	Low response
CRD 505	39.08	29	34.76	Moderate
CRD 500	35.88	27.34	31.24	Moderate
CRD 508	36.2	27.9	29.75	Low response
Jogesh	32.68	28.66	14.03	Low response
Pradeep	37.08	28.76	28.93	Low response
Asutosh	34.22	30	14.07	Low response
Jyotirmayee	35.62	27.02	31.83	Moderate
Hasanta	36.2	28.5	27.02	Low response
Prativa	37.2	29.6	25.68	Low response
NuaAcharmati	35.7	28.4	25.70	Low response
Upahar	42.1	28.5	47.72	Moderate
Mrunalini	39.6	28.68	38.08	Moderate
Tejaswini	38.9	28.7	35.54	Moderate
CV	**4.394**	**3.744**		
CD (0.05%)	**2.068**	**1.337**		

Very-low response: <10 per cent, Low response: 10-30 per cent, Medium response: >30 per cent.

metabolites present in the seeds of 16 varieties used in experiment. Results reported are similar with Vishwanath *et al.* (2013) in rice; Rai *et al.* (2019) in Indian mustard; Rakesh *et al.* (2019) in pigeon pea and Raut *et al.* (2019) in wheat.

KOH test

Based on color developed in seed soaked decant chemical solution, 16 varieties used in experimentation were grouped into six classes such as no color, pale yellow, yellow, deep brown or reddish brown, pale brown and brown as shown in (Table 4 and Fig. 1). Similar findings were also noted by Vishwanath *et al.*(2013); Singh *et al.*(2017); Rai *et al.*(2019); Raut *et al.*(2019); Rekesh *et al. (*2019).

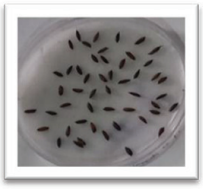
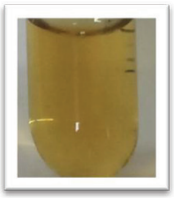
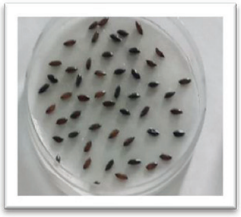
GA_3 test

Effect of gibberellic acid, a growth regulator on growth of seedlings over control was noted. The coleoptile length of rice genotypes showed varied response to GA_3 (Table 5). The highest increase in coleoptile length was observed in Manaswini (48.87 per cent) and the lowest was in Jogesh (14.03 per cent). Based on a percentage increase in coleoptile length over the control, eight varieties exhibited moderate response and another eight varieties exhibited low response to gibberellic acid. Similar findings were noted by Vishwanath *et al.* (2013); Raut *et al.*(2019) and Rakesh *et al.* (2019).

Effect of Gibberellic acid awas used to study seedling growth behavior for classification of rice varieties. During seedling response to GA3, the percent increase in coleoptile length was observed as Very low response: < 10 per cent, Low response: 10-30 per cent, Moderate response: > 30 per cent. Similar results were noted by Chakrabarty and Agrawal (1990) and Lee *et al.* (1992) in soybean; Raut *et al.* (2019) in wheat; Rakesh *et al.* (2019) in pigeon pea and Anitalakshmi *et al.* (2014) in rice.

Vishwanath *et al.* (2013) used chemical tests to identify and characterize 24 tomato cultivars, including the standard phenol test, modified phenol test, NaOH test, KOH test, and seedling growth response to additional chemicals. According to the research, most of the cultivars evaluated were found to be unique among other cultivars. However, distinct chemical properties were used as a key for cultivar identification, and to distinguish between all cultivars.Anitalakshmi *et al*. (2014) also found that among numerous chemical tests, phenol and modified phenol tests offered the most consistent findings and could be utilized to differentiate rice cultivars successfully. Nagendra *et al.* (2020) conducted research to find suitable chemical methods for identifying 25 popular rice varieties that are rapid, reliable, and simple for seed analyzers, breeders, and seed growers. The investigation demonstrated that phenol and modified phenol tests affected the colour of TKM 9 and TRY 1

Fig. 1 : Color of seed soaked decant to the various chemical tests

Variety	Phenol test	Modified phenol test	NaOH test	KOH test
Asutosh	Pale brown 	Brown 	Yellow 	Deep brown/reddish brown 
CRD-202	Pale brown 	Pale brown 	Yellow 	Pale yellow 
CRD-500	Brown 	Black 	Yellow 	Pale yellow 
CRD-505	Very deep brown 	Deep brown 	No color 	Pale brown 

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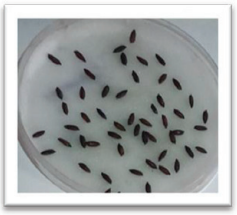
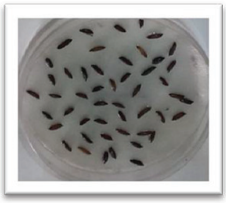
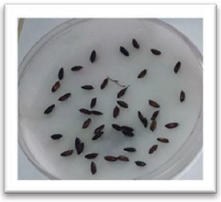
varieties to brown, while no colour change was seen in the variety I.W. Ponni.

For identification of cotton genotypes, the chemical tests for seeds such as sodium hydroxide and potassium hydroxide were found to be useful, while gibberellic acid tests for seedling response were found useful (Reddy *et al.,* 2008). Tiwari *et al*., (2013) reported using phenol, modified phenol and NaOH tests of seeds and GA_3 and 2,4-D tests of seedlings of four rice varieties. Ukani *et al*. (2018) investigated to study characterization of 25 sesame genotypes through chemical tests like NAOH test, KOH test and differential growth response of coleoptile length to GA_3 and seedling response to 2,4-D. Study showed that chemical tests and seedling response to GA_3, 2,4-D were found useful in the grouping of sesame genotypes. The NaOH test changed the color of rice variety (TKM 9) from colorless solution to red color. The GA_3 and 2,4-D tests classified the cultivars into two and three groups, respectively, based on shoot growth (Nagendra *et al*., 2020). Although no single chemical test was able to distinguish all the varieties, a combination of chemical tests was proved beneficial in varietal identification. The use of phenol, peroxidase, and NaOH tests for wheat varietal identification was reported, whereas the KOH test was ineffective (Ukani *et al*., 2016).

CONCLUSION

Testing of single variety or genotype using various systemic chemical test yields proper grouping which help in standardization of cultivar purity testing of new released varieties, whereas Phenol test is base model

CRD-508	Pale brown 	Brown 	Pale yellow 	Pale brown 
Hasanta	Brown 	Brown 	Pale yellow 	Yellow 
Hiranmayee	Very pale brown 	Deep brown 	Pale yellow 	Pale brown 
Jogesh	Brown 	Brown 	Pale yellow 	No color 

Contd....

for grouping of variety, remaining tests like Modified Phenol test, NaOH test, KOH test and GA_3help in further subgrouping of variety or genotype.

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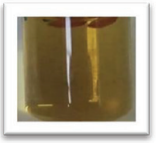
Nagendra, M.S., Selvaraju, P., Jerlin R., Ganesamurthy, K. and Senthil, N. 2020. Identification and

Jyotirmayee	Very deep brown 	Brown 	Yellow 	Reddish brown/ Deep brown 
Manaswini	No color 	Pale brown 	Pale yellow 	Brown 
Mrunalini	Very pale brown 	Pale brown 	Pale yellow 	Yellow 
NuaAdarmathi	Brown 	Brown 	Yellow 	Pale brown 

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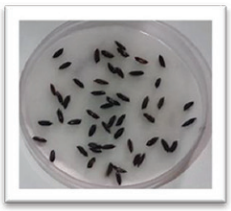
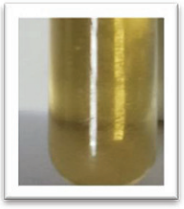
Sripunitha, A. and Sivasubramaniam, K. 2014. Varietal characterization of rice varieties based on chemical methods. *Trends in Biosciences,***7**(20) : 3139-3146.

Pradeep	Brown 	Black 	Pale yellowish 	Brown 
Pratibha	Pale brown 	Pale brown 	Yellow 	Brown 
Tejaswini	Deep brown 	Black 	Yellow 	Pale brown 
Upahar	Pale brown 	Pale brown 	Pale yellow 	Yellow 

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