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Distribution of genes producing phenol colour reaction in grains of wheat and its related species, mode of inheritance and breeding for low polyphenol activity

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Abstract

One of the important *chapati* quality traits pertains to the darkening of whole meal dough and its subsequent effect on browning of *chapatis* (unleavened Indian bread). The grain pericarp of most of the bread wheat varieties grown in India reacts to phenolic compounds and produce dark brown or brown colour. The present investigation undertook a most intriguing challenge to breed a variety, which do not react to phenol and does not darken the dough. A large number of wild wheat species, bread wheat cultivars, durum wheat and synthetic hexaploid (SH) wheat were screened to identify wheat genotype(s) with negative reaction to phenol. A variety of phenol reactions were recorded among the wheat species having different genomes. Most of the *Aegilops squarrosa* and *Triticum timopheevi* accessions showed negative colour reaction. Two phenol non-reactive (–ve) SH wheats (SH26 and SH32) were crossed with hexaploid bread wheat variety, HD 2851 with positive reaction (+ve) to phenol. Phenol test of F_1 grains reacted positively and produced dark brown colour on pericarp indicating that the phenol colour reaction is a dominant trait. The F_2 population segregated into two categories where the grains of 746 plants developed colour and 13 were non reactive (-ve colour or no colour). The Chi-square analysis revealed that development of phenol colour in grains is governed by three dominant alleles as the observed frequency of plants fit well into theoretical ratio of 63 (+ve) : 1 (–ve). The subsequent BC_1-F_2 and BC_2-F_2 generations were simultaneously tested with phenol to facilitate selection of the genotypes with –ve reaction. A few genetic stocks and selections derived from multi-parent-cross did not develop colour upon testing with polyphenol. These genetic stocks have been improved further in respect of other agronomic traits. In Addition, an alternative chemical to phenol was discovered, which gave comparable results, safer to use, would not affect germinability adversely and thus amenable to high through put application in breeding programme.

Key words: Polyphenol oxidase activity, wheat species, paracetamol, grain pericarp, phenol reaction, development of colour

Introduction

Wheat is one of the most important cereal crops globally as well as in India. Both hexaploid (*Triticum aestivum* L.) and tetraploid (*T. durum* Desf. and *T. dicoccum* Schulf.) wheat species are source of staple food for 30 per cent of the world's population (Eversole et al. 2015). Wheat is consumed in the form of bread, noodles, pasta, semolina and other wheat based products. Bread is the staple food of the western world, whereas in Eastern Asia 50% of wheat flour is consumed as noodles (Anderson and Morris 2001). Likewise in India, approximately 85% of the total wheat produce is being consumed in form of chapati which is the staple diet of Indians. An ideal *chapati* develops puffing, has typical aroma, creamish tinge, silky appearance and remains soft for five to six hours of storage. The issues on quality have attained much importance in last couple of years after India attained sufficiency with regards to wheat production. Recently, a surge in demand for good *chapati* quality wheat in Indian market is noticed. One of the important *chapati* quality traits pertains to the time-dependent darkening of whole meal dough and its subsequent effect on browning of *chapatis* and other end use products. This is the result of high levels of activity of enzyme poly phenol oxidase (PPO) present in the pericarp (bran layer) of wheat kernel (McCaig et al. 1999). Poly phenol oxidase (PPO) is a complex enzyme having both monophenolase and diphenolase activity (Fuerst et

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al. 2006). Monophenolase activity refers to hydroxylation of monophenols to *o*-diphenols (E. C. 1.14.18.1) and diphenolase activity refers to oxidation of *o*-diphenols to o-quinones (E. C. 1.10.3.2) (Steffens et al. 1994). These quinones in turn can react with nucleophiles like amines, thiols and phenolics forming complex dark coloured compounds called melanins (Whitaker and Lee 1995). It has been observed that a high PPO activity is responsible for whole meal dough darkening (Abrol et al. 1970). PPO activity can be assayed from wheat flour as well as whole wheat kernel (Hatcher and Kruger 1993 1997; Kruger et al. 1994; Fuerst et al. 2006).

When the water soaked cereal grains come in contact with aqueous solution of phenol, the pericarp of the grain(s) reacts and develop dark colour. The phenol colour reaction in wheat seeds was accidentally discovered by Pieper (1922) when he tried to disinfect seeds infected with common bunt with Betenal containing phenol. While studying the reaction, he noticed that colour of the seeds turned brown, dark brown and blackish or unchanged after a few hours. Later Hermann (1928) studied the characteristics of this phenomenon in detail and used it as a means to identify wheat varieties and their seed purity. In the subsequent years, phenol colour reaction test was established as standard seed testing procedure world over (Anon. 1966). Phenol colour reaction on outer glumes can also be used to distinguish varieties (Esbo 1945, Nair and Tomar 2001). With this evidence that the phenol reaction used as a method to distinguish varieties indicates that this trait is not affected with environment and therefore it has the potential to serve as an excellent genetic marker. Voss (1938) reported that the development of pigmentation is the result of conversion of phenol and other phenolic derivatives into melanin pigments in the presence of tyrosinase (Monophenol Oxidase). Researchers have also indicated that the grains that react with phenol to develop colour have the undesirable property of browning wheat products like pasta and noodles (Lamkin et al. 1981; Bernier and Howes 1994; Mahapatra et al. 1989), thus proving to be useful technique in screening the end products.

After establishing the validity of the test, several studies have been conducted since then to know the distribution the genes controlling phenol reaction. The PPO activity varies among wheat cultivars and also affected by environment (Baik et al. 1995; Park et al. 1997; Watanbe et al. 2006). The phenol colour reaction is governed by one or two genes in common wheat, the dark colour being dominant (Miczynski 1938), while Joshi and Benerjee (1969) reported that black reaction was dominant over both brown and white in emmer wheat. Further, they suggested that brown was dominant over white and thus concluded that a single gene has multiple alleles for this trait. Bhowal et al. (1969) suggested that the A genome contributed the gene controlling phenol reaction in hexaploid wheat. However, Demeke and Morris (2002) reported that durum (AABB genomes) cultivars develop significantly low amount of PPO activities. Nair and Tomar (2001) reported that there are at least two genes controlling phenol colour reaction in *Triticum turgidum* var. *durum* Desf. Zeven (1972) used substitution lines of common wheat Chinese Spring for location of gene(s) controlling phenol colour reaction. He identified that chromosomes 2A and 2D carry one gene each for this trait but did not exclude the possibility of another gene in the homoeologous chromosome 2B. Wrigley and McIntosh (1975) reported that the gene influencing the phenol colour reaction of grain was located on long arm of chromosome 2A. Earlier, Sharma et al. (1995) mapped a tyrosinase producing gene on chromosome 6B in common wheat. Several studies indicated that the genes for phenol colour reaction are widely distributed across the 3 genomes in wheat. Demeke et al. (2001) reported that the enzyme polyphenol PPO has been implicated in discolouration of Asian noodles is controlled by quantitative trait loci distributed on 2A, 2B, 2D and 6B chromosomes.

Reducing or eliminating polyphenol oxidase activity (PPO)-based browning is an important aspect of improving wheat quality. Although several measures can be adopted by the food industry to limit enzymatic browning, including the use of reaction inhibitors, physical treatments and the use of by produce extracts and modified atmosphere (Anderson and Morris 2001; Taranto et al. 2017), breeding varieties with negative phenol reaction or reduced amount of PPO activities is the best way. Most of the common wheats, particularly released varieties develop colour (darkening) when treated with phenol lowering dough quality and reducing commercial value of the product. Therefore, a study was undertaken to reduce PPO activity in common wheat through breeding and rapid screening based on phenol reaction. The mode of inheritance of genes controlling phenol reaction was also studied along with the screening of a large number of wheat species. This phenol test is popular among researchers, however it found limited application in large scale screening of segregating populations in a

breeding programmes on account of the test material (seeds) loosing germinability and phenol as such being potent health hazard. Hence, an experiment was also conducted aimed at finding alternative chemical(s) that are expected to give comparable results and would not affect germinability adversely and thus, may be amenable to high through put application in breeding programme.

Materials and methods

Plant materials

Two hundred and thirty one genotypes belonging to diploid (2x), tetraploid (4x) and hexaploid (6x) wheat species including the derivatives from the crosses were subjected to phenol test to study the distribution of colouration (phenol reaction) in *rabi* 2015-16. We have also used accessions of 18 species of *Triticum* genus and 16 species of *Aegilops* genus (Table 1). Out of 94 synthetic hexaploids (SH) screened for phenol reaction during the year 2008-09, six SH (SH 29, SH 35, SH 26, SH 32, SH 36 and SH 94) along with some bread wheat varieties were tested again for their reaction against phenol. Among the common wheat genotypes screened for phenol reaction (the term polyphenol oxidase (PPO) activity is also used) four varieties, *viz*., HD2851, HD2733, PBW343 and HD2932, which gave positive (+ve) reaction to phenol and two synthetic hexaploid SH 26 (ACO89/*Aegilops tauschii* (WX309:TA2454) and SH 32 (DOY1/*Ae. tauschii* (WX447) wheats with negative (-ve) reaction against phenol were taken for hybridization. Crosses were made between SH26 and HD2851, HD2733, PBW343 and HD2932. A cross involving multiple parents (HD2851/SH32//HD2851/F_3/T281/F_3/PBW343) was also made with the objective of improvement in agronomic traits. The population derived from SH 32 x HD 2851 only was considered for further breeding and genetic analysis in this communication. Based on the screening selection for desirable colour of the end product (dough) particularly for *chapati* quality was adopted.

Screening methodology

Five grains of parents, F_1 hybrids, BC_1F_2 and BC_2F_2 were taken for phenol test. Five grains from each plant were wrapped with absorbent paper and soaked in water for 16h at 20±1°C. After soaking the grains were unwrapped, blotted dry and placed on Whatman No. 1 filter paper in covered petri dishes moistened with freshly prepared 1% aqueous solution of phenol at 25±1°C. After 4h, the degree of darkening of grains was visually scored as, black/dark brown (3), brown (2) and light brown (1) and as no colour (0) reaction. No categories among the colouration of grains were made for the purpose of calculating Chi-square values. Instead, only two categories were made i.e., positive (+ve; colour development) and negative (-ve, no colour or vey light colour) reaction. The test was repeated to confirm the results.

A chemical named acetaminophen commonly known as paracetamol was also used as an alternative to phenol. Ten grains of wheat samples were kept in one ml of 2% aqueous solution of paracetamol dissolved in warm water and kept at 35°C for one hour. To recover the plants with negative phenol reaction backcrossing and selection in each F_2 generation (BC_1-F_2 and BC_2F_2) was followed. Test for seed germinability was undertaken in the grains of five varieties of bread wheat *viz.,* HD 2329, C 306, HD 2851, HI1500 and PBW343 and two durum cultivars PDW233 and HI 8498 after testing with 2% paracetamol and 1% aqueous solution of phenol (replicated twice). Grains were placed on blotting paper in petriplates and soaked with water. Observations on percentage of average seed germination was recorded 1h, 2h, 4h, 6h after phenol and paracetamol treatment after 16h soaking. For studying the inheritance, the F_1, F_2 and BC data were subjected to Chi-square tests for goodness of fit to expected ratios.

Results

Phenol reaction in diverse germplasm

It is axiomatic that varietal purity is a very important consideration in crops. Phenol colour reaction of grains is one of the several tests to ensure seed purity. Pericarp of the wheat kernel reacts to phenol and produce colour which is assessed to classify the grains based on different colour grades. It is unequivocally a characteristic of the genotype and hence serves as a marker. The gene(s) controlling phenol reaction (polyphenol oxidase activity) were well distributed among the wheat species. Phenol acts as substrate for the enzyme poly phenol oxidase present on seed coat and causes black colouration. The degree of colour development varies with different genotypes. In genotypes with good storing quality or low poly phenol oxidase activity, phenol could not create black colour. Reaction to phenol test revealed wide variation among the different accessions. Among the diploid species, the grains of all eight accessions of *Triticum monococcum* and five accessions of *T. urartu* (A genome) produced dark brown/black colour. Hence

Table 1. Phenol colour reaction in wheat species, common and durum wheat cultivars and some derivatives from HD2851*2/Synthetic hexaploid wheat

Gen./Acc. No.	Phenol reaction/grade			
	No colour (0)	Light brown (1)	Brown (2)	Dark brown/black (3)
Diploid species	***Aegilops speltoides*** (SS) Acc. -EC497604, IARI254 ***Ae. squarrosa*** (DD) Acc.- 3161, 3436, 3472, 3489, 3700,3735, 3743,3745,3747, 3750, 3751, 3753, 3758, 3759,3760, 3761, 3786, 3806, 5574, 9783, 9788, 9385, 9810, 9811, 9812, 9816, 9825, 9827, 13757, 13764, 13780, 14091, 14092, 14095, 14102, 14104, 14106, 14107, 14113, 14130, 14163,14195, 14197, 14199, 14215, 14219, 14221, 14226, 14227, 14228, 14230, 14231, 14234, 14239, 14240, 14244, 14249, 16252, 500516, DWR22, DWR32, DWR37 ***Ae. comosa*** (CC) Acc.- EC105985 ***Ae. sharonensis*** ($S^{sh}S^{sh}$) Acc.- IARI226 ***Ae. umbellulata*** (UU) Acc.- EC497610, EC497611	***Ae. squarrosa*** (DD) Acc.- 3742, 3784, 14222, 14231, W126 ***Ae. markgrafii*** (CC) Acc.- WLT 3249 ***Aegilops speltoides*** (SS) EC 383066, IARI118	***Ae. speltoides*** (SS) Acc.-WLT 1902, WLT2003, WL T2102, WLT2103, IARI84, IARI133, IARI 137, IARI236, IARI253 ***Ae. squarrosa*** (DD) Acc.- 3755, 9807, 14116, 14119, 14171, 14514, W162, DWR33 ***Ae. umbellulata*** (UU) Acc.-IARI2, IARI6 ***Ae. markgrafii*** (CC) Acc.-IARI230, IARI232	***T.monococcum*** (AA) Acc.-IARI64, IARI67, IARI109, IARI237, WLT 2801, WLT 2802, WLT 2804, WLT 2809 ***T. urartu*** (AA) Acc. -EC578096, EC 578249, WLT 4049, WLT4047, IARI242 ***Ae. speltoides*** (SS) Acc.- WLT2001, IARI63, IARI233,
Tetraploid species	***T. timopheevi*** (AAGG) Acc. IARI189WA, IARI190WD, IARI191WB, IARI192WC, IARI193WE, IARI235, IARI240 ***T. militinae*** (AAGG) Acc.- 1170001 ***T. pyramidale*** (AABB) IARI181 ***T. durum*** (AABB) cvs. MACS 1967, MACS2694, HI8627	***T.durum*** (AABB) *cv.* WH896, HI8591, HI8663, HI8381, Jayraj, RD10, RD20 ***T.dicoccum*** (AABB) cv. Khapli72 ***T. polonicum*** (AABB) Acc.- LSP5 ***Ae. kotschyi*** (UUSS) Acc.- WLT1001, WLT4054, IARI 131 ***Ae. variabilis*** (UUSS) Acc.- IAR I238 ***Ae. peregrina*** (UUSS) Acc.- IARI60 ***Ae. biuncialis*** (UUMM) Acc.-EC573205	***T. durum*** (AABB) cvs. HD4530, A206, Raj.3765, ***T. dicoccoides*** (AABB) Acc.- GSP13, ***T. turgidum*** (AABB) Acc.-MSP-6, ***T. polonicum*** Acc., LSP9, LSP10, ***Ae. geniculata*** (UUMM) Acc. EC 331765, ***Ae. neglecta*** (UUMM) Acc.-EC 573305, ***Ae. triuncialis*** (CCUU) Acc.-EC299352, ***Ae. biuncialis*** (UUMM) Acc.-EC105986	***T. durum*** (AABB) cv. Bijaga Yellow, DWR137, HI7483, HI8998, HI4672, Gulab, MACS9, NP404 ***T. dicoccoides*** (AABB) Acc.- IG 46386, IG46391 (ICWT600902), GSP3, GSP6, IAR I36, IARI39, IARI45 ***T. dicoccum*** (AABB) cvs. Mexican Dwarf 278. CTTR4013 ***T.turgidum*** (AABB) Acc.-IARI182 ***T. carthlicum*** (AABB) Acc.-IARI194 ***T. polonicum*** (AABB) Acc., LSP6, LSP8, IARI195 ***T. abyssinicum*** (AABB) Acc.-IARI180 ***Ae. variabilis*** (UUSS) Acc.- IARI88 ***Ae. triaristata*** (UUMM) Acc.- EC 383062 ***Ae. ovata*** (UUMM) Acc.- EC573309, EC 573311 ***Ae. geniculata*** (UUMM) Acc. EC497597, EC 497598
Hexaploid species	***Ae. juvenalis*** (DDMMUU) Acc.-EC 497599 Synthetic hexaploid wheat SH 29, SH 32, SH36 ***T. aestivum*** Derivatives from HD2581*2/SH, Derv. 3109, 3113, 3115, 3117, 3121; Selections from the multiple cross, Sel. 3137, 3139, 3141, 3149, 3153, 3155, 3157	***T. aestivum*** cvs. NI5439, K8027; Synthetic hexaploid wheat SH 94 ***T. spelta*** (AABBDD) Acc.- PI348494, IARI272 ***T. macha*** (AABBDD) Acc.- IARI185	***T. aestivum*** cvs. HD2888, HW2044, C306, HW2004, HD2781 ***T. compactum*** (AABBDD) Acc.-IARI245	***T. spelta*** (AABBDD) Acc.- PI348557, PI348528, PI348654, PI348764, PI348670, PI348597, PI348744, PI348495, PI348531, IARI267, IARI269, IARI270, IARI273, IARI276 ***T. amplissifolium*** (AABBDD) Acc. IARI102 ***T. vavilovii*** (AABBDD) Acc.-IARI188 ***T. aestivum*** cvs. HD2851, PBW343, HD2733, HD2932, HD2967, Sona-lika, WR544, HW2006, PBW621-50

Acc.= Accession; Gen. = Genotype

none of the A-genome donors were negative to the phenol test. In case of *Ae. squarrosa*, there was wide variation within the species. Out of 75 accessions, 62 showed no colour reaction, 5 showed light brown and 8 showed brown colour reaction. All *Ae. speltoides* accessions except two showed colour development, whereas, the only accession of *Ae. sharonensis* did not produce any colour (Table 1). Unlike the A and S genomes species, three accessions of *Ae. markgraphii* (CC) gave +ve reaction while *Ae. comosa* (MM) produced –ve reaction. In addition, another diploid species, *Ae. umbellulata* (UU), two accessions showed +ve reaction and rest two were negative. A wide distribution of phenol reaction among different species was recorded producing brown and light brown no colour reaction (Table 1).

Among the 42 accessions of tetraploid wheats carrying AABB genomes, most of the species, *viz*., *T. durum* (21), *T. turgidum* (2) and its sub species, *T. dicoccum* (3), *T. dicoccoides* (8), *T. carthlicum* (1), *T. pyramidale* (1) *T. abyssinicum* (1), *T. polonicum* (4) produced brown or dark brown colour except one accession of *T. pyramidale* and 3 cultivars of *T. durum* produced no colour. All the seven accessions of *Triticum timopheevi* Zhuk. (AAGG) and its mutant species *T. militinae* reacted negatively to phenol treatment and hence producing no colour. Tetraploid species having other than the AABB genomes, namely, three accessions each of *Ae. geniculata* (UUSS) and *Ae. kotschyi* Boiss. (UUSS), two accessions of *Ae. biuncialis* Vis. (UUMM) and *Ae. ovata*, and and one each of *Ae. triuncialis* L. (UUCC), *Ae. neglecta* (UUMM), *Ae. triaristata* (UUMM) and *Ae. peregrina* (UUSS) gave +ve reaction (Table 1). However, two accession of *Ae. variabilis* Eig. (UUSS) gave differential reaction one being positive and the other very light or negative colour. *Ae. juvenalis* (Thell.) Eig. (DDMMUU), the only hexaploid showed negative reaction.

Common wheats with ABD genomes *viz*., HD2851, HD2733, HD2932, HD2967, Chinese Spring and PBW343 produced dark brown or brown colour, whereas synthetic 6x wheats, SH 29 and SH 35 reacted positively producing light brown colour. However, SH 26, SH 32, SH 36 and SH 94 gave negative reaction. Hexaploid species namely, *T. vavilovi*, *T. amplissifolium*, *T. macha*, *T. spelta* and *T. compactum* showed +ve reaction with varied degree of colour. The reactions to acetaminophen (paracetamol) were also recorded and showed similar and comparable to polyphenol reaction.

Genetics of phenol colour reaction

The phenol treated grains of the *T. aestivum* varieties, HD2851, HD2733 and BW343, which were used as parents produced black or dark brown colour, while SH 26 and SH.32 produced negative reactions. The seeds obtained from F_1 of the crosses, HD2851 x SH. 26 and HD 2851 x SH 32 reacted positively producing black colour indicating that the black colour (+ve reaction) is dominant over no colour production (–ve reaction). To work out the mode of inheritance only two categories (coloured vs non-coloured) were considered for classifying the treated grains. In the segregating F_2 population derived from HD 2851 x SH 26, the grains of 759 single plants were tested for phenol colour reaction (Table 2). The phenol treated

Table 2. F_1 response and F_2 segregation for phenol colour reaction on grains

Cross	Generation	Phenol reaction		χ^2 value	P value
		+ve	-ve		
HD2851/SH 26	F1	15	0		
	F2	746	13	0.501	0.4791
HD2851/SH 32	F1	15	0		
	F2	4 89	9	1.19	0.2753

grains of 746 plants developed colour (reacted positively), whereas 13 did not develop any colour. This frequency of treated grains in F_2 generation gave a good fit to a expected theoretical Mendelian ratio of 63 (+ve) : 1 (–ve) (χ^2 = 0.501; P = 0.4791). Similarly, the grains obtained from F_2 derived from HD2851 x SH 32 segregated into positive (489 grains) and negative (9 grains) phenol reactions fitting well into a theoretical ratio of 63:1 with 1.19 χ^2 value (P: 0.2753) indicating that the phenol colour reaction is controlled by three genes.

Test for seed germinability

The grains of five varieties of bread wheat *viz*., HD2329, C306, HD 2851, HI1500 and PBW343 and two durum cultivars, HI 8498 and PDW233 were tested for phenol reaction with phenol and paracetamol. The seed germinability was tested after treatment with 2% paracetamol solution and 1% aqueous solution of phenol. The seed germination ranging from 92 to 96% in bread wheat and 86 to 96% in durum cultivars was recorded after 1h, 2h, 4h and 6h, respectively after paracetamol treatment, while under phenol test it

ranged from 0 to 60% in bread wheat, individually HD2329 recorded 60% after 1h, C306 (0%), HD2851 (28%), HD 1500 (7%) and PBW 343 (2%) (Table 3). The durum cultivars HI 8498 showed 8% while PDW 233 did not germinate after 1h treatment. The seeds of only variety HD 2329 survived (8%) for 2h and 4% after 3h (Table 3). Gradual reduction in seed germination of bread wheat varieties under phenol treatment was recorded in a separate test showing 92% germination after 15 minutes, 89 after 30 minutes, 28 after 1h and zero per cent after 1.5h except HD 2329. However, after 23h, seed germination was affected badly under paracetamol treatment. Contrasting picture of seed germination after treating the grains with phenol and paracetamol is given in Fig. 1.

Breeding for –ve phenol colour reaction

The phenol treated grains of different wheat species and common wheat varieties and the stocks used as parents for introgression of non-reactive trait were scored for colour. Synthetic wheats, which did not develop any colour were considered as –ve, while HD2851, HD2733 and PBW343 reacted positively and developed black colour on pericarp. The grains of F_1 derived from the reciprocal crosses also produced positive reaction and developed black colour similar to its parents indicating that the positive phenol colour is dominant over non-reactive grains. A schematic presentation of the breeding for negative phenol colour reaction. All the F_1 hybrids were partially hard threshing and therefore, we forwarded the population from SH

Table 3. Effect on seed germination of selected wheat varieties (in per cent) of 1% phenol and 2% paracetamol solution after 16h of soaking

Genotype	Treatment	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour	6 Hour	Water	23 Hour
HD2329	Phenol	**60**	**8**	**4**	0	0	0	98	0
	Paracetamol	96	96	96	96	95	95		68.5
C306	Phenol	**0**	0	0	0	0	0	96	0
	Paracetamol	96	96	96	95	93	93		37.5
HD2851	Phenol	**28**	0	0	0	0	0	97	0
	Paracetamol	96	93	92	92	92	91		85.7
HI 1500	Phenol	16	0	0	0	0	0	96	10
	Paracetamol	96	0	0	0	0	0	95	41.6
PBW343	Phenol	**2**	0	0	0	0	0	96	0
	Paracetamol	95	94	94	94	93	92		81.4
HI8498 (d)	Phenol	**8**	0	0	0	0	0	96	0
	Paracetamol	96	92	96	92	-	-		11.4
PBW34 (d)	Phenol	0	0	0	0	0	0	88	0
	Paracetamol (socked16h)	87	87	87	87	86	86		11.4

d= Durum; -=Not recorded

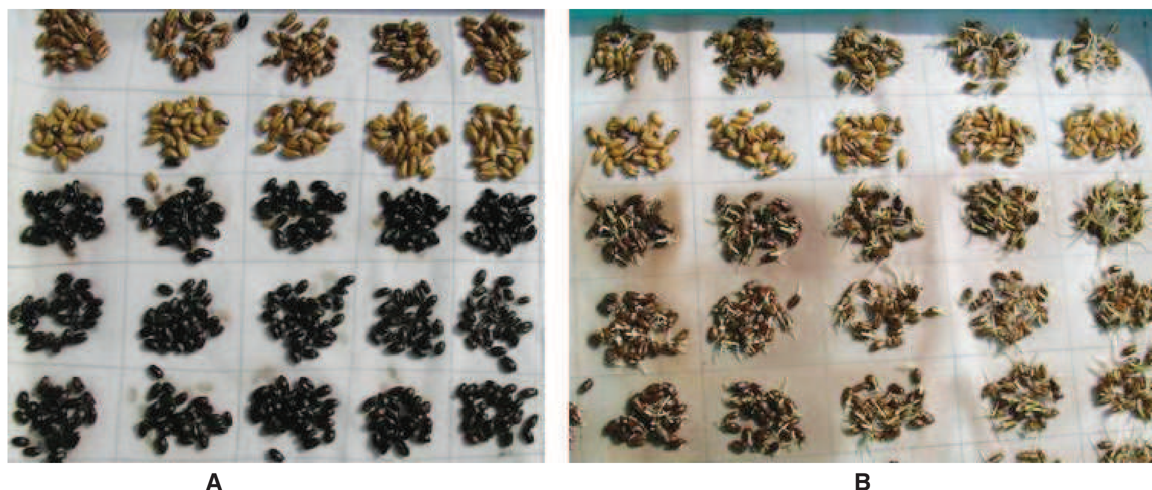

Fig. 1. Depiction of phenol colour development by treating wheat grains with phenol and paracetamol. A = No germination after phenol treatment, B = Germination after paracetamol treatment

32 x HD 2851, which had comparatively easy threshing and the grains were amber in colour. The F_2 grains (498) obtained from selfed F_1 plants were tested with phenol and out of nine only an amber grained plant (No. 17) was selected and backcrossed to HD2851 in next season. From the progeny derived from Plant 17 x HD2851 a single plant from BC_1-F_1-F_2 with negative phenol reaction was selected and further backcrossed with HD2851 in next season. The obtained BC_2F_1 was raised and selfed to obtain F_2 grains. The F_2 grains from 1467 plants were again tested with phenol. From the BC_2F_2 segregants, seven plants, which did not develop any colour were selected. The plant No. 49 with negative reaction and amber grain colour was backcrossed to HD2851 and obtained BC_2F_1 seeds were raised to get BC_3F_2 segregating population. After testing the grains of individual F_2 plants, finally six single plants with negative reaction were selected.

Discussion

Polyphenol oxidase activity cause undesirable browning of wheat products such as, pasta, noodles and whole meal dough subsequently affecting *chapati* quality and reduce commercial value. Polyphenol oxidase activity has been extensively studied with respect to physico-chemical properties also (Vaughan and Duke 1984; Fronk et al. 2015). PPO isoenzymes can be distinguished based on electrophoretic mobility, optimal temperature, pH and substrate specificity. Wheat shows an optimum of 5.3 to 6.9 pH (Interesse et al. 1980). The genes controlling phenol colour reaction are well distributed across the genomes of wheat species and their distribution is evidenced by the reaction of wheat species tested in the present study. Among the diploid wheats, all the accessions of *T. monococcum* and *T. urartu* developed black colour displaying high PPO activity. Most of the *T. dicoccoides* and some other tetraploid sub-species having AABB genomes and several accessions of *T. aestivum* (AABBDD) also produced black colour of similar intensity of *T. monococcum* and *T. urartu* containing ancestral form of PPO activity characterized by A genome. Interestingly, durum wheat varieties show limited variation in phenol colour reaction and have significantly lower PPO activity than bread wheat cultivars in spite of having the PPO genes (Demeke and Morris 2002; Pasqualone et al. 2014). Further analysis is required to find out the presence of PPO genes and their activity in durum cultivars. The results obtained in present study supported earlier findings (Bhowal et al. 1969; Fuerst et al. 2008) that the A genome present in tetraploid and hexaploid species is contributed by either of the two species, *T. monococcum* or *T. urartu.* Out of 16 accessions of *Ae. speltoides* tested, 3 produced black/brown colour (+ve reaction), two developed light brown, whereas two did not produce any colour (–ve reaction). Variation in colour development indicated that the locus got mutated at diploid level and both alleles producing colour and not producing colour were contributed to tetraploid wheats. The present results are contradictory to earlier reports of Bhowal et al. (1969) and Prakash et al. (1991) who tested a small sample but in agreement with Fuerst et al. (2008) who reported a high PPO activity in *Ae. speltoides* contributing the S/B genome to tetraploid wheat with AABB genome.

No accession of *Triticum timopheevi* (AAGG) and its mutant species *T. militinae* reacted to phenol treatment indicating that the genomes A and G/S involved in the evolution of *T. timopheevi* did not carry the allele which reacts to phenol or produces tyrosinase or its substrate in pericarp. Another possibility is that the genes responsible to synthesize or accumulate the particular enzyme lost during the course of evolution. But 8 accessions of *T. monococcum* and 5 accessions of *T. urartu*, tested with phenol reacted positively and produced black or dark brown colour signifying that the species contributed A genome to *T. timopheevi* does not exist in the present germplasm. Whereas the other tetraploid wheats (AABB) inherit the A genome from either of the tested species possessing the positive allele for phenol reaction and it is well distributed. However, the non-reactive grains of durum in high frequency got selection preference in nature. Since, the progenitors produce positive polyphenol oxidase activities, there is no possibility of elimination/deletion of genes during evolution through mutation. The situation is making us believe that the gene(s) responsible for producing positive reaction got mutated or eliminated during the course of evolution of *T. timopheevi* or It is also likely that genome S contributed by *Ae. speltoides* suppresses the gene(s) present in a genome of *T. timopheevi*. A few accessions of *Ae. speltoides* also reacted positively to phenol treatment. However, a large number of accessions need to be tested.

Generally, the grains of most of the *T. aestivum* germplasm from India produce tyrosinase in the pericarp and reacts with phenol and produce colour, the non reactive genotypes are rare. Singhal and Prakash (1988) tested 82 Indian bread wheat genotypes in aqueous solution of phenol and based on phenol reaction categorized them into four groups

as black, dark brown, brown and light brown colour; none of the genotype was non reactive (negative reaction) to phenol. A large number of notified Indian durum wheat varieties have been characterized for phenol reaction by different researchers. Kundu et al. (2006) reported only 7 varieties out of 44 with –ve reaction (no PPO activity); the remaining ones reacted positively with dark (10), medium (4) and light (23) phenol reaction, whereas Rajender et al. (2009) tested 57 wheat cultivars among which, six cultivars viz., Raj 3765, HD 2888, NI 5439, HD 2781, PBW 533 and HI 1500 showed weak phenol colour reaction, but possessed high PPO activity (*Ppo-A1a*) and low PPO activity (*Ppo-D1a*) alleles on chromosome 2A and 2D, respectively. Two genotypes *viz*., MP 4010 and DL 788-2, with low PPO activity (*Ppo-A1b*) and high PPO activity (*Ppo-D1b*) alleles showed strong phenol reaction. These results indicated that intensity of the colour development may not have correlation with the degree of PPO activity.

Phenol reaction of grains and glumes in 132 accessions comprising diploid, tetraploid and hexaploid species has also been reported by Singhal et al. (1991). In earlier study, we tested 90 synthetic hexaploid wheats (*T. turgidum* ssp. *durum* X *Aegilops squarrosa*) developed at CIMMYT (data unpublished) and found that SH 26, SH 32, SH36 and SH94 gave –ve reaction producing no colour or very light brown colour when tested with phenol. Bhowal et al. (1969) tested four synthetic wheats *viz*., *dicoccum-squarrosa*, *dicoccoides-squarrosa* (2) and *carthlicum-squarrosa* of which only *carthlicum-squarrosa* produced black colour upon testing with phenol. A synthetic hexaploid wheat (M2) developed at CIMMYT by crossing a tetraploid wheat Ruff (*Triticum turgidum* ssp. *durum* Desf.) with *Aegilops squarrosa* (Coss.), accession no. 112 was identified to have very low levels of polyphenol oxidase activity. Several researchers have assayed PPO enzymatic activities in wheat and reported, several genes/Qtls and multiple paralogous and orthologous PPO genes (Raman et al. 2005; Beecher et al. 2012, Hystad et al. 2015). It is obvious that the phenol reaction in the synthetic hexaploids is apparently dependent on the reaction of the varieties (species) entered in their synthesis

While studying origin and evolution of phenol colour reaction Bhowal et al. (1969) reported that the gene(s) conferring phenol reaction originated from the A genome in emmer and common wheat. Zeven (1972) provided evidence for two genes in hexaploid wheat located on 2A and 2D chromosomes. Nair and Tomar (2001) genetically analysed the grains of tetraploid and hexaploid wheat and reported that tetraploid wheat varieties Bijaga Yellow and HD4530 each carrying single gene which are non-allelic indicating two independent genes. Fuerst et al. (2008) determined the PPO in different diploid species and reported that high PPO activity is associated with chromosome 2A of *T. monococcum* being an ancestral form. Report on low magnitude of PPO activity in *Ae. speltoides* indicated that B genome also carry gene(s) for phenol reaction (Fuerst et al. 2008). They also proposed an additional loci contributing to PPO activity is located on 7B chromosome (PI481521). Earlier, Watanabe et al. (2006) mapped two genes *Tc1* and *Tc2* for high phenol colour reaction on 2A and 2B chromosomes, respectively in durum wheat. A gene for high PPO activity has also been mapped on 2D chromosome by Jimenez and Dubcovsky (1999) in Chinese Spring. Recently, Sadeque et al. (2017) studied mode of inheritance of polyphenol oxidase activity in doubled haploid population derived from a hybrid between VAW08-A17 and QALBis and reported PPO activity is controlled by three genes located on 2AL, 2BS and 2DL (additive mode of action) chromosomes in wheat.

Since the positive phenol reaction of pericarp in wheat grains is controlled by three dominant alleles, conventional breeding of genotypes with non-reactive grain is time consuming. After each F_2 generation, selection of plants having grains with non-reactive pericarp produced by a recessive genotype need to be backcrossed with recurrent parent. The population size needs to be large to select desirable plants with good agronomic traits. Also, the testing of a large number of F_2 segregants is cumbersome. Selected recessive plant was backcrossed with recurrent parent (HD2851) and the BC_2-F_2 grains were again subjected to phenol test. The process was repeated till a segregant producing amber grain colour with good size and non-reactive to phenol from BC_3-F_1-F_2-F_3 generation. Finally, four derivatives, namely, Derivatives, 3113, 3115, 3117 and 3125 derived from SH32 x HD2851. Four selections (Sel.) viz., Sel. 3137, 3139, 3141 and 3149 were taken for using in breeding programme. Low PPO lines have been earlier reported by other researchers, which contained novel allele at Ppo-A2 and null allele at Ppo A1 and Ppo-D1 loci (Hystad 2014).

Testing of seed material with paracetamol is safer and if the tested seeds need to be planted to grow the next generation. The treated seeds of tested bread wheat and durum varieties germinated (>91%

and 86%), respectively after 6h of submergence into the paracetamol solution, whereas the germination of treated seeds in phenol reduces drastically after an hour and completely stopped after one and a half hour except one variety. Even though phenol test is popular among breeders it reduces seed germinability and is a dangerous chemical itself. Hence, an alternative chemical acetaminophen commonly known as paracetamol which has phenolic base produced comparable results not affecting germination is safer to apply in breeding programme for quick screening.

Authors' contribution

Conceptualization of research (SMST, V, BS, MN); Designing of the experiments (BS, MN, NM, SKJ); Contribution of experimental materials (BS, V, MN, NM); Execution of field/lab experiments and data collection (MN, BS, AA, AV, SKJ); Analysis of data and interpretation (SMST, BS, V, MN); Preparation of manuscript (SMST, V, MN, SKJ, NM).

Declaration

The authors declare no conflict of interest.

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