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An improved *in vitro* germination medium for recalcitrant bread wheat (*Triticum aestivum* L.) pollen

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Abstract

There is no robust and quick technique available for testing germination of wheat pollen. In this study, Brewbaker and Kwack (BK) pollen germination medium (PGM) was adjusted with α-amino caproic acid (EACA), peptone water and maltose to develop an improved medium for wheat. Germination of pollen in this PGM ranged from 92% to 98.2% depending upon genotype pollen germination media (PGM). This is the first report of PGM which has >95% wheat pollen germination *in vitro*.

Key words: Recalcitrant wheat pollen, peptone water, maltose, ε-amino caproic acid (EACA), *in vitro* pollen germination

Introduction

Pollen is a deceptively simple, semi-autonomous male gametophyte that functions to deliver sperm cells to the ovule and enable fertilization. Wheat pollen belongs to grass family *Poaceae,* in which pollen grains are shed in hydrated state (30-35%) as compared to 1-5 % of the pollen of orthodox species. They lose viability when moisture level is brought down by either drying or desiccation and hence they are classified as recalcitrant pollen. The Unorthodox pollen does not undergo maturation drying, are prone to desiccation, shed at high moisture content and germinates immediately (Franchi et al. 2011). These characteristics posed difficulties in earlier attempts to germinate wheat pollen (MacFaddin 1985; Cheng and McComb 1992). In wheat, to date, there is only one report and a patent available on *in vitro* pollen germination. Cheng and McComb (1992) reported a raffinose enriched medium supporting 81.7 % pollen germination with 35% pollen tube bursting after 1 h. A patent (2014) (CN 103627668 A) entitled "*In-vitro* wheat pollen germination method" claims not less than 80% germination rate at 28^{o}C in the medium used.

Here, we describe a reproducible pollen germination medium that allows unprecedented levels of pollen germination in a species belonging to "dry stigma" type. Three chemicals *viz*., å-amino caproic acid (EACA), peptone water and maltose have been used which are conventionally not the regular components of pollen germination medium. Peptone water is a minimal growth medium that contains peptone as carbon source along with nitrogen, vitamin, sodium chloride and other minerals. It is mainly used for cultivation of non-fastidious microorganisms (MacFaddin 1985), carbohydrate fermentation and production of indole (Balows et al. 1991). å-amino caproic acid (EACA), an amino acid derived from *n*-caproic acid (*n*-hexanoic acid), a saturated 6 carbon fatty acid ($C_6H_{12}O_2$,$CH_3(CH_2)COOH$) occurring in milk fats, has been used to improve solubilization of membranes. It has been used in studies on overcoming self-incompatibility and incongruity in *Vigna* (Baker et al. 1975; Chen et al. 1978). Maltose as osmoticum, mostly sucrose has been preferred as better carbon source for pollen germination (Chen et al. 1978; Brewbaker and Kwack 1963). It was also hypothesized and tested that peptone can promote

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pollen germination and pollen tube growth based on its effect on mycelial growth of fungus.

Materials and methods

The study was conducted during 2011-2015 at Indian Agricultural Research Institute (IARI) Regional Station, Wellington, Tamil Nadu, India and wheat variety Agra local was primarily used.

Pollen collection and culture

Pollen was collected from appropriate ear heads by cutting off half the spikelet. Once anther started dehiscence, the pollen was dusted directly on to the germination medium by gentle tapping and cultured according to the pollen germination media (PGM) droplet method (Jayaprakash et al. 2013). All observations on pollen germination and pollen tube growth were recorded 15 min after incubation at 17±1°C for wheat pollen.

Assessment of pollen viability by staining

Two staining methods *viz*., a simplified Alexander staining (Peterson et al. 2010) and Fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison 1970) were used to assess viability. In Alexander staining, viable pollen stained deep blue and non-viable pollen stained pale blue. The fluorochrome FDA scored pollen grains that fluoresced brightly as viable and others as non-viable.

In vitro pollen germination and pollen germination media (PGM)

In this study, Brewbaker and Kwack (BK) medium (1963) was used as a basal medium. In a preliminary investigation, media having Brewbaker and Kwack medium salts, different concentrations of maltose (10, 20, 30 and 40 %) and/or 15 % polyethylene glycol (PEG) 6000 were screened to select a medium with appropriate medium constituents. The study was designed into 4 experiments as follows:

1. A 3x3x3 factorial experiment was designed with 3 concentration of Maltose (30, 27.5 and 25 per cent, three level of Boric acid (75, 60 and 50 mg l^{-1}) and three levels of Calcium nitrate (100, 200 and 300 mg l^{-1}).

2. To improve pollen tube growth and contain the bursting, media constituents were altered one by one using maltose, PEG 6000, EACA and/or peptone water to develop a complete pollen germination medium.

3. *Effect of incubation temperature and pH:* Pollen was incubated at different temperatures between 13-26^{o}C at 1^{o}C interval in the best PGM. The pH of the above medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Observations were recorded for pollen germination and pollen tube growth.

4. *Varietal response to PGM:* To validate PGM, few wheat varieties were randomly selected and pollen viability tested using the new PGM and its variants containing different levels of Peptone and/or EACA.

Statistical analysis

This experiment was conducted according to factorial based on completely randomized design with 5 replications. Data were analyzed by statistical analysis software SAS version 9.3 using analysis of variance (ANOVA) and differences among means were determined for significance at $P < 0.05$ using LSD test.

Results and discussion

PGM droplet technique

Using the PGM droplet technique (Fig. 1), pollen germination of wheat varieties *viz*., Pusa 4, LR14A, Agra Local, HW 5207, Thew, HW 5216 (this use of other varieties must be mentioned in materials and methods) etc. were initially tested before selecting Agra Local for further studies. Also, it helped to fix the initial levels of inorganic salts level for factorial experiments. Each season, at least 200 fresh media were prepared and tested. This allowed large number of media to be tested in a single day, thus avoiding preparation of fresh medium each time and furthermore, more than two genotypes or species could be tested simultaneously. This technique was found better than the usual liquid medium with microslides and solid medium used for pollen culture (Jayaprakash and Sabesan 2013.).

Assessment of pollen viability by staining

Alexander stain and FDA fluorochrome clearly demarcated between viable and non-viable grains (Fig. 2). During the cropping season, it was observed that sterile pollen count was between 0.36 to 5.2 percent (data not shown) and sterility increased (upto 12%) when there were intermittent rains.

In vitro pollen germination

Maltose x boric acid x calcium nitrate factorial experiment

Variance over initial factorial treatment (3x3x3) for

wheat pollen indicated highly significant differences for all the treatments associated with different media constituents (Table 1). All interaction effects between different media were significant (Table 2). Among the 27 combinations tested, two combinations were significant than others; the first combination (A1B3C1) which contains maltose (30%), BA (50 mg l^{-1}) and CN (100 mg l^{-1}) showed 98.79 % pollen germination which is on par with second treatment (A2B2C1) 27.5 % Maltose, 60 mg l^{-1} BA and 100 mg l^{-1} CN giving 98.44 % pollen germination. Based on pollen bursting and consistency of pollen tubes the later was selected for further studies.

Role of different medium components

Maltose as Osmoticum

Among different maltose concentrations (18,19, 20….27%) tried, pollen germination was highest (99.51) at 19% maltose followed by 99% at 18% maltose which is at par with other concentrations except 27%, 24% and 21% (Fig. 3). Hence, in further treatments 19% Maltose was uniformly used. Mostly sucrose has been preferred as better carbon source for pollen germination *in vitro* of many species (Shivanna and Rangaswamy 1992; Brewbaker and Kwack 1963). Sucrose is known to inhibit callus and androgenesis in wheat and barley, whereas maltose supported a high level of callus induction (Orshinsky et al. 1990; Hunter 1987). In our studies sucrose failed to give germination beyond 30% (data not shown) even when different PEG concentrations were included in the medium. Medium with 19% maltose supported consistent pollen germination (>99%) with a mean tube length of 750 µm after 25 minutes of incubation for wheat variety Agra local. A range of 295-713 µm length of pollen tubes were observed for different varieties within 20 minutes of culture as compared to 200 µm length reported after 1 hour (Cheng and McComb 1992). They reported an agar medium containing 0.75 M raffinose, 100 mg l^{-1} H_3BO_3, 300 mg l^{-1} $CaCl_2.2H_2O$ supporting 81.7% pollen germination, 35% pollen tube bursting Maltose was earlier preferred as better carbon source

Table 1. Mean sum of squares from analysis of variance for wheat pollen germination

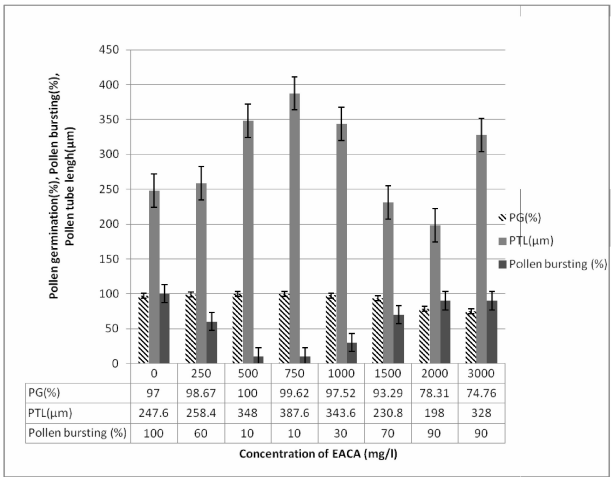
Source	df	Mean sum of squares pollen germination
Treatment	26	329.66**
Maltose (A)	2	469.75**
Boric Acid (B)	2	642.72**
Calcium Nitrate (C)	2	256.42**
A x B	4	273.75**
B x C	4	251.43**
A x C	4	302.88**
A x B x C	8	315.14**
Error	27	0.0641**

*CD (0.05%) = 0.173; significance at P = 0.01; CD (0.01%) = 0.231

Table 2. Interaction effects on pollen germination (%) under different maltose, boric acid and calcium nitrate combinations

Combination	C_1	C_2	C_3
A_1B_1	95.83^{de}	94.77gh	95.13fg
A_1B_2	97.71^{b}	91.06bl	96.39c
A_1B_3	98.79a	97.70b	97.61b
A_2B_1	95.77de	94.48b	96.53c
A_2B_2	98.44a	95.38ef	90.90l
A_2B_3	93.44i	59.40o	82.56n
A_3B_1	82.44n	95.58ef	90.18m
A_3B_2	96.13cd	97.71k	94.28hi
A_3B_3	91.84k	92.53g	39.44p

where A_1 = 30% A_2 = 27.5% A_3 = 25% of Maltose
B_1 = 75 B_2 = 60 B_3 = 50 mg l^{-1} of boric acid
C_1 = 100 C_2 = 200 C_3 = 300 mg l^{-1} of calcium nitrate
*similar letter indicates statistically on par bar on LSD



	0	250	500	750	1000	1500	2000	3000
PG(%)	97	98.67	100	99.62	97.52	93.29	78.31	74.76
PTL(µm)	247.6	258.4	348	387.6	343.6	230.8	198	328
Pollen bursting (%)	100	60	10	10	30	70	90	90

Fig. 3. Effect of EACA on pollen germination and pollen tube growth shows optimum concentration at 500 or 750 mg l^{-1}

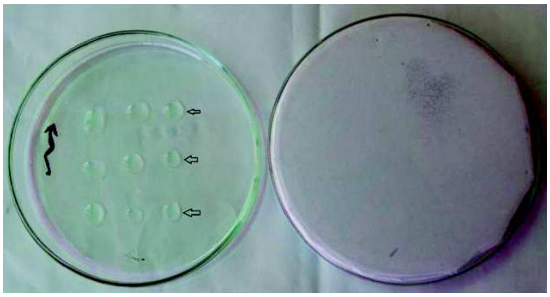


Fig. 1. PGM droplet technique showing a 3 x 3 media combination. (Arrows indicates medium droplets)

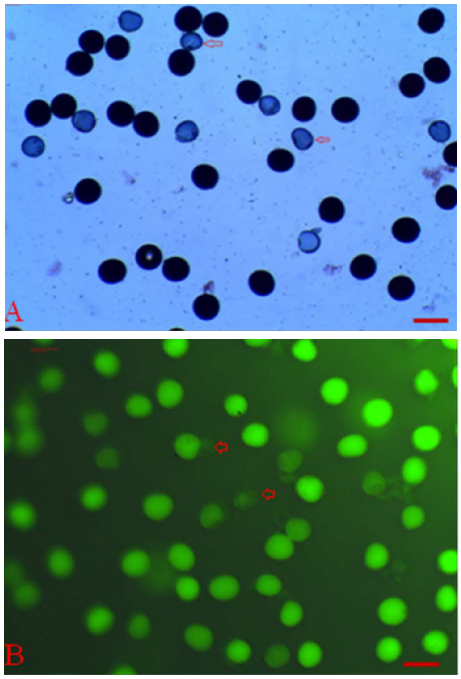


Fig. 2. (A) Alexander staining of wheat pollen (B) FDA staining. Viable pollen stained darkly (Arrows indicate the sterile pollen). Scale bar: 42 mm

in PGM than sucrose for *Eucalyptus marginata* (Wheeler and McComb 2006.). Use of maltose reduces the cost to 1/31th as compared to raffinose.

Polyethylene glycol 6000 supplemented medium

At 13% PEG concentration maximum of 99.58% pollen germination was observed followed by 99.31 at 14% PEG concentration. Though pollen germination was good, there was a reduction in pollen tube length at increasing concentration. However, even 13% PEG concentration showed pollen tube bursting. The role of PEG in pollen germination medium was well established and choosing appropriate molecular weight is the key for specific crop (Jayaprakash and Sabesan 2013.).

Boric acid and Calcium nitrate on pollen germination

When reduced level of boric acid was tested at 5 different levels, the 50 mg BA concentration showed 93.32% germination with a mean pollen tube length of 368 µm followed by 10 mg l^{-1} concentration (88.63% pollen germination with mean pollen tube length of 363 µm). Similarly for calcium nitrate, pollen germination was highest at 30 mg l^{-1} (99.46%) followed by 0 CN (98.66) and 10 mg (95.95). The pollen tube was longest at 30 mg l^{-1} (57.44) followed by 50 mg l^{-1} (45.88) and 20 mg l^{-1} (42.60). The role of boron and calcium on *in vitro* pollen germination has been well documented. Boron deficiency leads to pollen tube bursting as its required in the pollen wall structure (Acar et al. 2010). Pollen germination involves many ions with Ca^{2+} as the key player (Leonie Steinhorst and Jorg Kudla; 2013). Various combination among different media constituents resulted in the development of a medium (M19) which consists of 19% Maltose + 13% PEG 6000 + 50 mg l^{-1} boric acid + 30 mg l^{-1} calcium nitrate + BK salts + 1% agar. This medium resulted in >95 pollen germination. However, pollen tubes of all genotypes including Agra local showed pollen tip bursting.

Effect of e-amino caproic acid (EACA)

Among the EACA levels tested, both 500 mg and 750

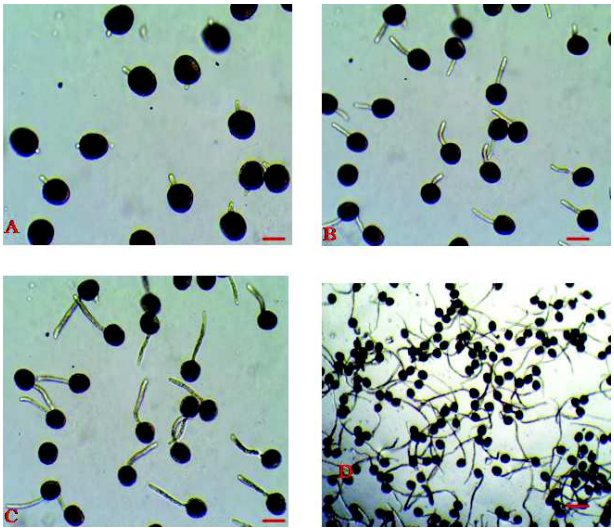


Fig. 6. Photomicrograph showing the pollen germination and smooth intact pollen tube growth of variety Agra local in medium M19 + 500 mgl^{-1} EACA + 100 mgl^{-1} Peptone + BK salts + agar (A) Arrows indicates budding of pollen after 2 minutes (B) smooth pollen tubes after 5 minutes (C) after 10 minutes and (D) after 15 min after culture

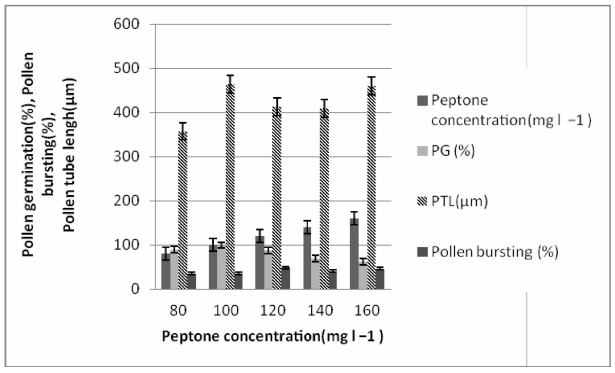


Fig. 4. Effect of peptone shows 100 mg l^{-1} peptone shows maximum pollen germination and pollen tube growth

mg l^{-1} concentration showed > 97% pollen germination and a mean pollen tube length of about 350 µm with 10% of pollen tube bursting (Fig. 4). Beyond this concentration there was a reduction in pollen germination and pollen tube length with increased pollen bursting. EACA is an immunosuppressor and was first used in PGM. It helped to establish complete PGM for pigeonpea. EACA might have played an important role in pollen germination by increasing lipid availability by membrane solubilization (Jayaprakash and Sarla 2001).

Effect of Peptone water

An improved pollen germination and pollen tube growth was seen at concentration 80-120 mg l^{-1}. The highest pollen germination was observed at100 mg l^{-1}peptone (99% PG; 349.6 µm PTL) followed by 120 mg of 87.22%, 387.6 µm respectively (Fig. 5) Peptone has been used in plant tissue culture for various effects such as induction of shoot regeneration in avocado (Nhut et al. 2008), promotion of hairy root formation in ginseng (Sivakumar et al. 2005) etc. Addition of peptone, contained pollen tube burst to a great extent, it was less than 20 per cent as compared to >85 % in the medium devoid of peptone however, the level of peptone varied with genotypes.

Effect of EACA and Peptone water

The pollen germination was complete (> 97%) at 100

Table 3. Percentage pollen germination and pollen tube length (µm) of some wheat genotypes in best PGMs along with response in M19 medium

Variety	Pollen germination medium	Pollen germination (%)	Pollen tube length(µm) (Mean ±S.E)	Range for pollen tube length(µm)
HD2833	M19 + 500 E	97.9 ± 0.047	418.8 ± 15.39	334.37- 713.00
	M19 + 750E	94.32 ± 3.67	667.7142 ± 32.18	
	M19 +500 E + 100 P	97.51 ± 2.98	553.75 ± 23.14	
	M19 + 750 E + 100 P	95.38 ± 3.09	334.37 ± 18.39	
	M19 + 500 E + 120 P	97.12 ± 1.87	680.00 ± 24.255	
	M19 + 750 E + 120 P	97.22 ± 1.74	713.0 ± 21.55	
HW 2044	M19 + 500 E	98.02 ± 1.03	265.60 ± 9.88	265.60 -467.50
	M19 + 750E	98.01 ± 0.076	467.5 ± 9.55	
HW 5207	M19 + 750E	98.0 ± 0.043	313.529 ± 12.45	246 -350.62
	M19 + 500 E + 120 P	98 ± 0.054	350.625 ± 14.91	
MACS 6145	M19	98.11 ± 0.87	360.58 ± 12.05	360.58 -603.13
	M19 + 750 E + 100 P	97.21 ± 1.54	603.13 ± 22.48	
	M19 + 750 E + 120 P	97 2.63 ± 2.11	546.25 ± 26.89	
HW 971	M19 + 500 E	98 .13 ± 1.42	462.50 ± 18.76	462.50 -551.25
	M19 + 500 E + 100 P	98.0 ± 0.66	551.25 ± 25.85	
HW 741	M19	98.23 ± 0.042	403.75 ± 21.13	241.87 – 403.75
	M19 + 500 E	97.23 ± 1.99	241.87 ± 8.55	
HW 1085	M19 + 500 E	98.18 ± 1.11	244.375 ± 11.44	
HW 1095	M19 + 500 E	98.01 ± 0.43	268.75 ± 11.83	190 -371.87
	M19 + 750 E + 100 P	92.91 ± 4.53	371.87 ± 16.36	
	M19 + 500E + 120 P	90.82 ± 4.76	278.125 ± 18.13	
	M19 + 750 E + 120 P	96.37 ± 3.75	190 ± 3.65	

P = peptone; E = EACA

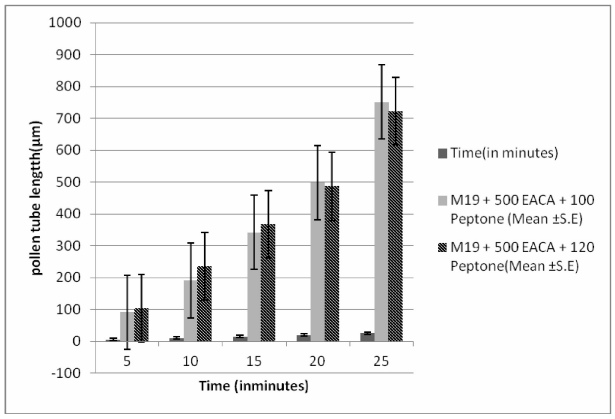


Fig. 5. Pollen tube length (µm) of wheat variety Agra local in 2 media (*M19 + 500 EACA + 100 peptone & M19 + 500EACA + 120 peptone*) at five minutes interval

mg peptone with 500 or 750 mg EACA concentration. In these media the pollen tubes were smooth and long (Fig. 6A-D) and there was reduced pollen tube bursting. It was observed that the pollen germinates within 5 minutes and pollen tubes reached 104 µm in medium with 120 mg l^{-1} peptone and as much as 751 µm pollen tube length after 25 minutes. At both100 mg l^{-1} and 120mg l^{-1} concentrations of peptone water, pollen germination and pollen tube length were good, but 100 mg peptone was preferred because of its consistency.

Effect of incubation temperature and pH

The experiment on incubation temperature showed 80-97 percent pollen germination in the temperature from 15-20^{o}C and the appropriate temperature of 17^{o}C showed maximum pollen germination of 97.8 % and a mean pollen tube length of 190 µm followed by 18^{o}C with 91.6% and pollen tube length of 170 µm. Pollen germination occurred at a lowest temperature of 13^{o}C (70.7%) and continued up to 26^{o}C (34.3 %). Outside the temperature regime pollen failed to respond.

The maximum pollen germination occurred at pH 6 with >95% pollen germination and mean pollen tube length of 280 µm. Pollen bursting was seen in pH below 5.5 and as the pH increased beyond 6, the pollen germination and mean pollen tube length was reduced. In pH 6.5, 7, 7.5 and 8.0 pollen germination was reduced to 40-60 % with 100-130 µm mean pollen tube length. The pollen germination rate was found to be higher in the pH range of 5.7-5.9 and it was appropriate at pH 5.9 where wheat variety agra local showed maximum germination >95% with intact pollen tube (345µm). Based on germination rate, burst rate and pollen tube length, the appropriate temperature reported for *in vitro* pollen germination of pecan pollen was 25^{o}C (Burke et al. 2004), for *Cajanus cajan* was 20.5 ± 2^{o}C (Jayaprakash and Sarla 2001), for *Cajanus cinereus, Rhycosia rothii* and *R. aureus was* 24^{o}C (Jayaprakash and Sabesan 2013). The pH of the germination medium has been shown to affect pollen germination and optimum pH for pollen germination of several species was rather narrow, with the best result generally at pH 7.3 or 8.3 and the poorest at 5.3 (Burke et al. 2004; Mbogning et al. 2011). The appropriate pH for wheat pollen is 5.9 as it showed more than 95% pollen germination with intact tube length (345 µm) at this level.

Varietal response to PGMs

The medium supporting more than 90% pollen germination were selected for each genotype though some of them showed pollen tube bursting which indicated the need for fine tuning of the medium (Table 3). The genotypes HW 971 and HW 741 responded well in M19 giving >95% pollen germination and pollen tube length of >400µm. This is adjudged as the best medium since it did not require supplementing either EACA or peptone. It is noted that some genotypes (HD 2833, HW 971, HW 1095 etc.) require EACA alone in PGM whose response were at par with medium having EACA + peptone. Some varieties such as MACS 6195, HW 1095 etc. showed better responses (>92% germination) in PGM with a combination of EACA (500 or 750 mg) and peptone water (100mg). These varieties responded poorly when peptone water was increased beyond 100 mg which reduced the pollen tube length.

Among the varieties tested, two of them *viz*., HD 2833 and HW 1095 showed more that 94 percent in all the media tested. The genotype HD 2833 showed the maximum pollen tube length of 713 µm in PGM with 750 mg l^{-1} EACA and 120 mg l^{-1} peptone and the pollen of HW1095 achieved a mean pollen tube length of 190 µm in the same medium The variety HD 2833 showed a mean pollen tube length in the range of 334.37 to 713 µm followed by MACS6145 with 360.58 to 603.13 µm and HW671 with 462.50 to 551.25 µm for mean pollen length. Similar kind of genotypic differences for pollen germination requirement have also been reported (Jayaprakash and Sarla 2001).

It is concluded that wheat pollen viability can be assessed quickly by staining with FDA or simplified

Alexander stain. The germination capacity of pollen of any bread wheat genotypes can be tested in the PGM reported here. It was observed that nitrogen source of peptone helped to promote smooth and intact pollen tubes by arresting pollen tube bursting as its role played with mycelia growth of fungi.

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