Indian J. Genet., 77(1): 25-31 (2017)
DOI: 10.5958/0975-6906.2017.00004.9

A reproducible *in vitro* pollen germination medium for recalcitrant cereal rye pollen (*Secale cereale* L): Effect of tryptone

P. Jayaprakash*, D. Sheeba, V. K. Vikas, M. Sivasamy, Jagdish Kumar, S. Annapoorani and Navaneetha Krishnan

ICAR-Indian Agricultural Research Institute, Regional Station, Wellington 643 231, The Nilgiris, Tamil Nadu

(Received: March 2016; Revised: December 2016; Accepted: December 2016)

Abstract

A reproducible *in vitro* pollen germination medium (PGM) for cereal rye is reported here using tryptone for the first time. Rye pollen is very difficult to germinate on nutrient medium being trinucleate and is classified as recalcitrant pollen. This study addresses the possibility of testing the viability of rye pollen within short time (15-20 min) using pollen germination medium (PGM). The viability of rye pollen was tested with FDA staining also. The results of both staining and *in vitro* pollen germination were found comparable. In this study, tryptone, a nitrogen source was used in addition to essential sugar and inorganic salts. PGM used for rye consisted of 19% maltose, 13% poly ethylene glycol 6000, 62.5 mgl^{-1} boric acid, 50 mgl^{-1} tryptone and 1% agar. During the flowering period, a pollen sterility of 3-12% was recorded. The PGM showed >93% pollen germination with intact pollen tubes at 17±1^{o}C and pH 6-7. This tool would be useful in many pollen biotechnological aspects such as pollen transformation, overcoming crossability barriers especially in wheat x rye wide hybridization etc.

Key words: Rye, *in vitro* pollen germination, maltose, recalcitrant pollen, tryptone

Introduction

Cereal rye is closely related to wheat and barley and is grown primarily in Eastern, Central and Northern Europe. It is more hardy grain, more tolerant to frost and drought than wheat. Establishing a pollen germination protocol and pollen germination medium is a prerequisite for attempting any pollen biotechnology such as studying gene expression (Zhou et al. 2015; Higashiyama and Takeuchi 2015), isolation and manipulation of sperm cells (Lu et al. 2015); overcoming crossibility barriers (Nadeem et al. 2015) and cryopreservation of pollen (El-Homosany and Hossam 2015). This is especially useful in wheat X rye hybridization as it has played a vital role in wheat improvement. For example, 1B/1R translocation in wheat carried many resistance genes including *Sr31* (stem rust), *Lr26* (leaf rust), *Yr 9* (stripe rust) and *Pm8* (powdery mildew) (Mirzaghaderi 2010). Recently, Nguyen et al. (2015) reported that addition of 4R chromosome to wheat increased anther length and pollen grain number. Rye has some useful traits which are desired in wheat restorer line such as anther extrusion, long anther, large amount of pollen and long pollen viability. Transfer of these traits to wheat may make the hybrid bread wheat, a reality.

Grass pollen is shed at hydrated state and very difficult to germinate artificially. They lose viability when moisture level is brought down by drying or desiccation and hence, fall into the category of recalcitrant pollen (Franchi et al. 2011). In any pollen germination medium a sugar, boric acid, calcium, magnesium and potassium salts are commonly incorporated. This medium is often enriched with polyethylene glycol, amino acids, etc to perfect a standard PGM for any crop species. Besides the media components, external factors such as temperature, pH, and relative humidity also play vital role in setting up conducive conditions for pollen germination in medium. The rain and the sun light on particular day also affect pollen germination. Pollen being sensitive to all the factors makes *in vitro* pollen germination results often inconsistent (Shivanna and Rangaswamy 1992).

*Corresponding author's e-mail: jpsarit@gmail.com
Published by the Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
Online management by indianjournals.com; http://epubs.icar.org.in/journal/index.php/IJGPB

Earlier, pollen germination in rye homozygotes and heterozygotes for 21 different reciprocal translocations showed highest of 93.6% by pollen collected from a single plant which was heterozygous for translocation however the overall mean pollen germination of 74.5 per cent (Figueiras et al. 1985). The PGM used in the study consisted of 100 ml water, 3.5g sucrose and 20mg boric acid at pH 6.5. Another report, with pollen germination medium of 20g sucrose, 10^{-3} M boric acid, 10^{-3} calcium nitrate and 1% agar at 24^{o}C at 96% RH could achieve 76.3% pollen germination for rye (Shivanna and Harrison 1981).

Here, we describe a viable protocol to test viability of rye pollen. In this PGM (1) Maltose instead of mostly used sucrose has been preferred as better carbon source for pollen germination (2) It was hypothesized that tryptone, a nitrogen source used in germination medium of microorganisms can promote pollen germination and pollen tube growth, based on its effect. It was added to PGM to examine its effect on pollen germination and pollen tube growth. By replacing or subtracting the components we provided new insights into pollen germination kinetics and mechanism of pollen tube growth.

Materials and methods

The study was conducted at Indian Agricultural Research Institute (IARI) Regional Station, Wellington, Tamil Nadu, India, during 2013-2015.

In vitro pollen germination and pollen germination media (PGM)

In this study, inorganic salts such as boric acid, calcium nitrate, magnesium sulphate and potassium nitrate were used. Maltose was used as osmoticum in place of sucrose. In a preliminary investigation, agarified media having inorganic salts, different concentrations of maltose (10, 20, 30 and 40 %) and/or 15 % polyethylene glycol 6000 were screened to select a medium with appropriate medium constituents. A 3x3x3 factorial experiment was designed with 3 concentrations of Maltose (18, 19 and 20 per cent, three level of Boric acid (62.5, 75 and 80 mgl^{-1}) and three levels of polyethylene glycol (PEG) 6000 (12,13 and 14 per cent). With all these medium 30 mgl^{-1} calcium nitrate + 100 mgl^{-1} magnesium sulfate + 200 mgl^{-1} potassium nitrate was included. A medium showing maximum pollen germination with minimum bursting was selected and different concentrations of tryptone (0, 25…100 mgl^{-1}) were added to standardize the medium.

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 medium by gentle tapping and cultured according to the PGM droplet method (Jayaprakash and Sabesan 2013). All observations on pollen germination judged by pollen tube length greater than the diameter of the pollen grain and pollen tube length (PTL) were recorded after 15 min incubation. Pollen was incubated in best PGM at different temperatures to see its effects on pollen germination and pollen tube growth. The pH of the above medium was adjusted from pH 5.0 to 8.0 with 0.1N HCL and 0.1N NaOH and observations were taken.

Since there were intermittent rains which resulted in inconsistent results, the plant was grown in two different environment (1) poly-house (2) open field. The effect of medium constituent was tested for both conditions to develop a reproducible pollen germination medium for both the condition.

Pollen viability by FDA staining

The rye pollen viability was tested by Fluorochromatic Reaction (FCR) Test (Heslop and Heslop 1970). The bright green or yellowish green fluorescence observed under UV pollen grains were considered viable and non viable grains fail to fluoresce brightly

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

In this study, it was observed that rye pollen germinated in a simple medium consisting of maltose, PEG 6000, boric acid and tryptone. There has been reports of unsatisfactory pollen germination with pollen belonging to the family of *Poaceae* (Franchi et al. 2011). Rye pollen measured 60µm in diameter with 28-33% moisture level as compared to 42µm of wheat pollen with 30% moisture content. The pollen viability lasts for 2.5 h since pollen shedding as compared to wheat pollen which loses viability in less than 30 minutes. To exploit heterosis in wheat, the restorer line of wheat has to be manipulated in traits such as anther extrusion, long pollen viability etc. Translocation of 4R chromosome of rye has shown to increase pollen

number and longer anther length in wheat (Nguyen 2015). In this context, testing the viability of wheat and rye pollen would be a useful tool in wheat x rye wide crosses. Recently the author has developed a pollen germination medium for the foremost cereal crop wheat (Jayaprakash et al. 2015). Here, we describe the *in vitro* pollen germination in rye.

In vitro pollen germination

Sucrose (20-30%) in the initial medium gave inconsistent results with >30% pollen germination and bursting (data not shown) and the medium were subsequently substituted with maltose. Variance over initial factorial treatment (3x3x3) for rye pollen indicated highly significant differences for all the treatments and all interaction effects between different media components at P≥0.01 per cent for both pollen germination and pollen tube growth (Table 1A & 1B). Based on pollen bursting, initially a medium with 19% maltose +75 mgl^{-1} boric acid + 14 % PEG 6000+30 mgl^{-1} calcium nitrate + 100 mgl^{-1} magnesium sulfate + 200 mgl^{-1} potassium nitrate was selected. This medium showed 77% pollen germination and a mean pollen tube length of 250µm. However, after 20 min of incubation almost all pollen tube burst. Among different maltose concentrations (18,19, 20….27 per cent) tried, pollen germination was highest at 19% Maltose with 78.23% pollen germination with 297.34 µm pollen tube length (PTL) followed by 20% maltose (74.56% with >250.32µm PTL) (Fig. 1A). As maltose concentration increased, pollen grains did not show any increase in pollen tube growth and in decreased maltose concentration maximum pollen grains were bursting and hence, in further treatments 19% Maltose was maintained. Maltose seems to be better osmoticum as it has also supported wheat pollen germination (Jayaprakash et al. 2015).

Inclusion of polyethylene glycol 6000 improved the pollen germination by stabilizing osmotic level. Pollen germination was 83.23 % with 373.67 µm PTL at 13% PEG and decreased to 74.91 % with 340µm PTL at 14% PEG (Fig. 1B). Though pollen germination was good, there was uneven growth of pollen tube at varying concentration. However, even 13% PEG concentration showed pollen tube bursting. Different species responded well with PEG of different molecular weight. PEG 6000 at 13% concentration supported maximum rye pollen germination with maltose. The role of PEG in pollen germination medium was well documented.

Table 1A. Mean sum of squares from Analysis of Variance for rye pollen germination

Source	d.f.	Mean sum of squares pollen germination
Treatment	26	87.80**
Maltose (A)	2	501.51**
Boric Acid (B)	2	152.87**
Calcium Nitrate (C)	2	350.95**
A x B	4	7.82**
B x C	4	18.89**
A x C	4	14.39**
A x B x C	8	13.48**
Error	26	1.41**

CD (0.05%) = 0.4795; CD (0.01%) = 0.6472; **significance at P≥0.01

Table 1B. Mean sum of squares from Analysis of variance for rye pollen tube length

Source	d.f.	Mean sum of squares pollen germination
Treatment	26	587.44**
Maltose (A)	2	1737.08**
Boric Acid (B)	2	1948.47**
Calcium Nitrate (C)	2	2111.41**
A x B	4	518.14**
B x C	4	2224.33**
A x C	4	56.61**
A x B x C	8	60.40**
Error	26	0.64**

CD (0.05%) = 0.3236; CD (0.01%) = 0.4367; **significance at P≥0.01

Different combination between inorganic salts indicated more salts are not necessary for rye pollen to germinate. Then, salts were removed one by one and at the end, calcium nitrate, magnesium sulphate, and potassium nitrate were eliminated completely from the medium as they caused bursting and bulging of pollen tubes (*data not shown*). When reduced level of boric acid was tested at 5 different levels, pollen germination of 88% with 475µm pollen tube length was observed at 75 mg concentration (Fig. 1C). Various combination between different media constituents resulted in the development of a medium which consists of 19% Maltose + 13% PEG 6000 + 75 mgl^{-1} boric acid + 1% agar. This medium showed

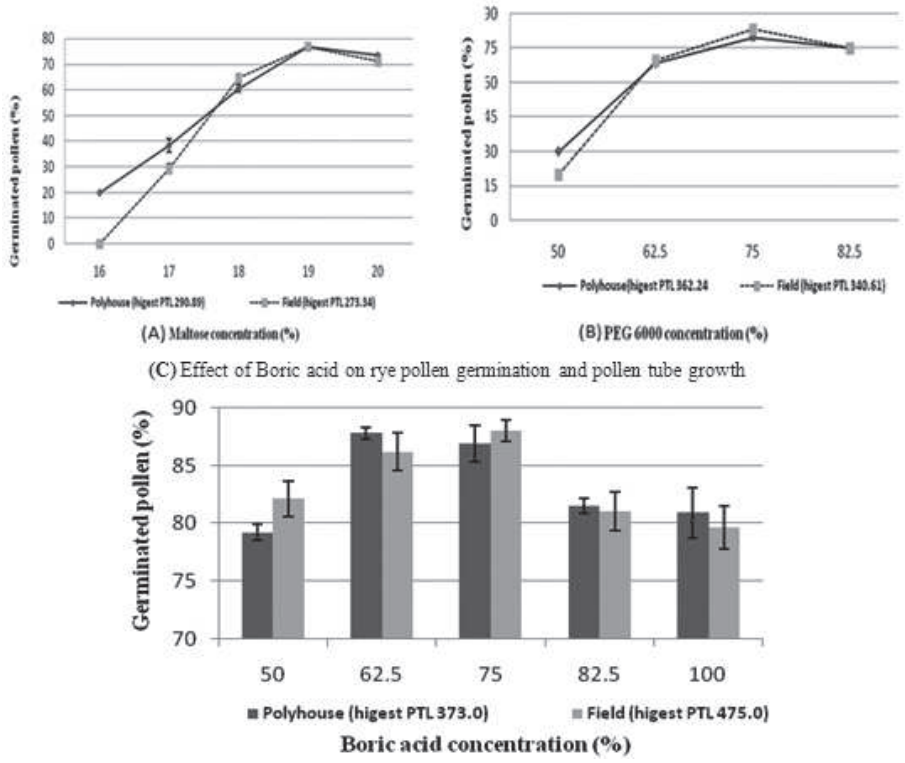

Fig. 1. Effect of (A) Maltose and (B) Poly ethylene glycol 6000 on rye pollen germination and pollen tube growth

88 % pollen germination. However, pollen tubes burst after germination and as high as 55% pollen bursting was seen.

To control pollen tube bursting, tryptone was added in the PGM. Analysis of variance for tryptone treatment showed significance at Pe≥0.01 per cent level. Among the tryptone levels (0-100mgl^{-1}) tested at 75 mg tryptone the pollen germination was 93.87 percent with pollen tube length of 512.50 µm followed by 50 mg concentration with 85.74% pollen germination and PTL of 484µm (Table 2). Use of tryptone supplemented media controlled the pollen tube burst completely and the pollen tubes were smooth and intact (Fig. 2A-D). Casein hydroxylate, an organic supplements used in pollen germination medium

Table 2. Effect of tryptone on rye *in vitro* pollen germination and pollen tube growth

Concentration of tryptone (mgl^{-1})	*Polyhouse collected pollen (Mean±S.E)		**Field collected pollen (Mean±S.E)	
	Pollen germination (%)	Pollen tube length (µm)	Pollen germination (%)	Pollen tube length (µm)
0	74.01±1.17	175.0±8.75	70.43±1.83	234.0±0.98
25	81.32±1.17	422.5±13.37	79.16±1.46	230.0±0.94
50	91.30±0.86	539.7±17.08	79.74±1.85	184.0±5.82
75	79.69±2.78	380.5±12.04	85.87±1.78	312.0±0.81
100	79.87±1.76	269.6±8.53	81.26±1.41	381.0±1.01

was found to promote pollen germination of *Arabidopsis thaliana* (Rodriguez et al. 2011). In wheat, peptone water at 100mgl^{-1} concentration along with e-aminocaproic acid (500-750 mgl^{-1}) enhanced smooth pollen tube growth and showed 95% pollen germination (Jayapraksh et al. 2015). Tryptone was good growth-stimulating nitrogen sources in the cultivation of *Trichoderma hamatum* and *T. harzianum* (Bianca et al. 2009). It was found to be the best organic nitrongen source for kefiran production by *Lactobacillus kefiranofaciens* (Dainiel et al. 2015), asparaginase production from *Enterobacter cloacae* (Anjana and Islam 2015), production of biosurfactant by *Bacillus subtilis* (Gudina et al. 2015). In this study, tryptone at 50-75 mg concentration in the medium it gave satisfactory and reproducible level of rye pollen germination. The complete pollen germination medium for rye consists of 19% Maltose + 13% PEG 6000 + 75mgl^{-1} boric acid + 75 mg tryptone + 1% agar. (SM 1 *A-D.mpeg*: *video clips showing rye pollen germina-tion*).

This medium was used to find appropriate temperature for rye pollen germination *in vitro,* temperature was varied from 12 to 26^{o}C (Fig. 3A). The experiment on incubation temperature showed >70% percent pollen germination in the temperature between 14-20^{o}C and the appropriate temperature of 17^{o}C showed maximum pollen germination of 91.35% with pollen tube length of 476 µm followed by 85.19% pollen germination with

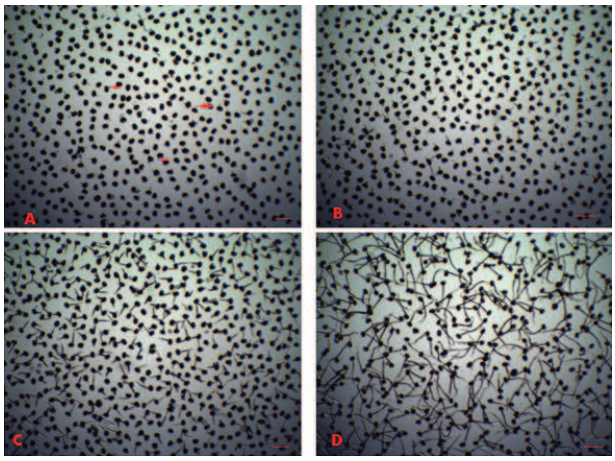

Fig. 2. Photomicrograph showing the pollen germination and smooth intact pollen tube growth of variety rye pollen in medium M19 + 13% PEG 6000 +, 75 mgl^{-1} boric acid + 50 mgl^{-1}tryptone + 1% agar 500 mgl^{-1}. A=Initiation of pollen germination – Budding (Arrows); B=Pollen germination after 5 minutes of incubation; C=Elongating pollen tube after 10 min of incubation; D=Germinated pollen after 15 min of culture (Scale bar: 42 µm)

occurred at pH 7.0 with 90.65 % pollen germination and mean pollen tube length of 420µm was observed. A pollen germination of 85% was seen in pH range between 6.0 to 7.0 (Fig. 3B).

The grasses pollen is shed at higher moisture level and most of them are reported to have short viability (Shivanna and Johri 1985). The moisture content rye pollen was 28-33% and wheat pollen with 30% moisture at the time of shedding. Attempts to bring down the moisture content by desiccation showed loss in the viability. It could not tolerate even 10 min of desiccation. This trait posed difficulty in taking up pollen storage in these species.

FDA staining

The results of FDA staining and *in vitro* pollen germination were comparable (Fig. 4A & B). The pollen sterility in rye was recorded using FDA staining and *in vitro* pollen germination and it was observed the pollen sample showed sterility of 3-12% during flowering period.

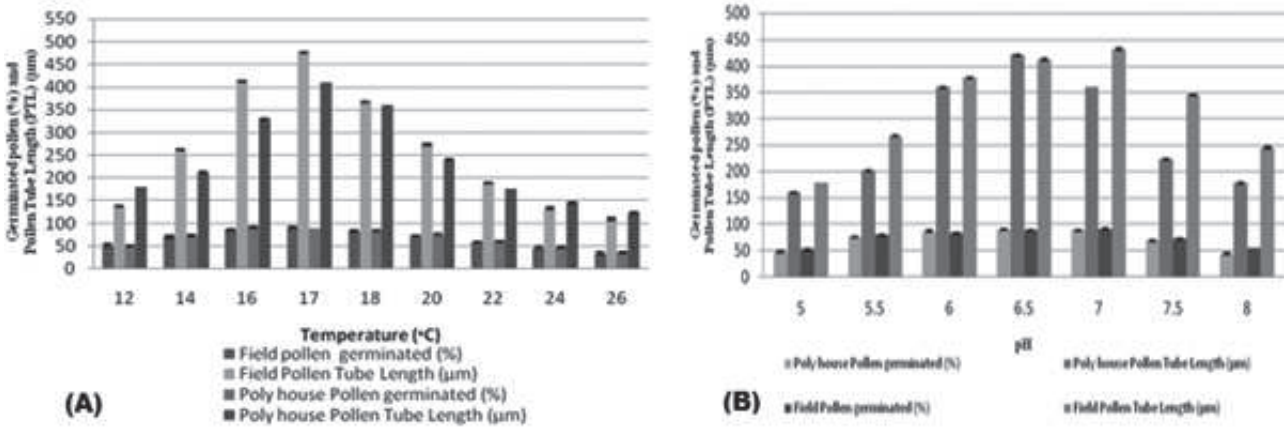

Fig. 3. Effect of (A) temperature on rye pollen germination and pollen tube growth and Effect of (B) pH on rye pollen germination and pollen tube growth

412 µm PTL at 16^{o}C. The appropriate temperature for rye pollen germination *in vitro* is 17±1^{o}C. The pollen germination drastically reduced below 14 and above 20^{o}C. Beyond 20^{o}C pollen tubes started bursting after 10 minutes of germination. Temperature is a crucial factor at various stages of reproductive growth of plants. Temperature stress reduced seed yield in many crops, for example *Brasicca* Young et al. (2004). Pollen is also very sensitive to temperature and it affects significantly pollen germination and pollen tube growth (Kakani et al. 2005). Maximum pollen germination

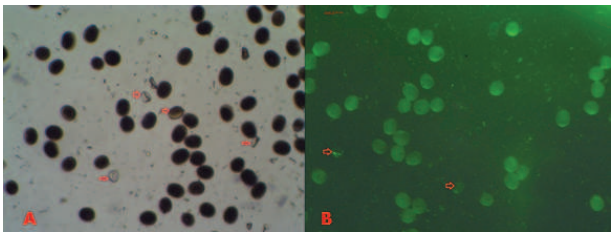

Fig. 4. (A) Rye pollen : Viable pollen with intact cell wall and appear dark whereas the sterile ones are transparent and shriveled and (B) FDA staining showing viable pollen stained darkly (Arrows indicate sterile pollen): Scale bar: 42 µm

If sterility of rye pollen is taken into account, the reported pollen germination medium (PGM) would definitely support more than 95% rye pollen germination. The viability of freshly shed rye pollen was above 95% and gradually decreased for every 30 min. After 2.5h the germination of pollen which maintained at room temperature showed below 45% with 50% pollen tube bursting.

The earlier attempts to germinate rye pollen *in vitro* have reported a maximum of 76% per cent pollen germination only with simple medium (Shivanna and Heslop 1981; Figueiras et al. 1985; Chaudhury and Shivanna 1987). In this study, nutrient requirements and conditions were standardized to germinate rye pollen which is a consistent protocol. The organic supplement, tryptone has proved to be a useful nutrient for rye in pollen germination *in vitro*. The pollen germination experiments with pollen from poly house showed that it required less concentration of boric acid concentration (62.5mgl^{-1} boric acid) and tryptone (50 mgl^{-1}) as compared to BA of 75 mgl^{-1} and tryptone 50 mgl^{-1} for field pollen.

Using the pollen from poly house and field conditions, it is concluded that pollen germination test can also be done during unfavorable environment conditions with minor adjustment in the media constituents. The rye pollen has relatively higher viability retention as compared to wheat and it may last for 2.5h. This provides opportunity to take up pollen storage experiments (cryopreservation) and *in vitro* pollen germination may be used to test the viability of fresh/ stored pollen. It would also be a viable tool in transfer of pollen traits through wheat x rye hybridization to wheat.

Authors' contribution

Conceptualization of research (PJ, DS, SA); Designing of the experiments (PJ, DS, SA); Contribution of experimental materials (PJ, MS, VK, JK); Execution of field/lab experiments and data collection (PJ, DS, NK, VK); Analysis of data and interpretation (PJ, DS); Preparation of manuscript (PJ, DS, VK, JK, MS).

Declaration

The authors declare no conflict of interest.

References

Anjana Sharma and Islam Husain. 2015. Optimization of medium components for extracellular glutaminase free asparaginase from *enterobacter cloacae*. Int. J. Cur. Microbiol. Appl. Sci., **4**(1): 296-309.

Bianca Caroline R. R., Brochetto-Braga M. R., Tauk-Tornisielo S. M., Carmona E. C., Arruda V. M. and Netto J. C. 2009. Comparative growth of *Trichoderma* strains in different nutritional sources, using bioscreening C automated system. Brazilian J. Microbiol., **40**: 404-410.

Chaudhury R. and Shivanna K. R. 1987. Differential responses of *Pennisetum* and *Secale* pollen. Phytomorphol., **37**: 181-185.

Daniel J. D., Elsayed A. E., Norzalina O., Roslinda A. M., Solleh R., Mohamad R. S., Ramlan A., Mohamad A. W., Hesham A. and Enshasy E. 2015. Development of cultivation medium for high yield kefiran production by *Lactobacillus kefiranofaciensi*. Int. J. Pharmacy Pharm. Sci., **7**(3): 159-163.

El-Homosany A. A. and Hossam A. S. 2015. Effect of Low Temperature and Cryopreservation on *in vitro* Pollen Germination of Some Olive Cultivars, American-Eurasian J. Agric. Environ. Sci., **15**(9): 1803-1808.

Figueiras A. M., Candela M. M. and Lacadena J. R1. 1985. Pollen germination and tube growth in rye (*Secale cereale* L.) homozygous and heterozygous for reciprocal translocations. Theor. Appl. Genet., **69**(5-6): 659-63.

Franchi G. G., Piotto B., Nepi M., Baskin C. C., Baskin J. M. and Pacini E. 2011. Pollen and seed desiccation tolerance in relation to degree of developmental arrest, dispersal, and survival. J. Exp. Bot., **62**(15): 5267-81.

Gudina J. E., Elisabete C. F., Rodrigues A. I., José A. T. and Lígia R. R. 2015. Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium. Frontiers Microb., 6-59.

Heslop-Harrison J. and Heslop-Harrison Y. 1970. Evaluation of Pollen Viability by Enzymatically Induced Fluorescence; Intracellular Hydrolysis of Fluorescein Diacetate. Biotech. Histochem., **45**(3): 115-120.

Higashiyama T. and Takeuchi H. 2015. The Mechanism and Key Molecules Involved in Pollen Tube Guidance. Ann. Rev. Plant Biol., **66**: 393-413.

Jayaprakash P. and Sabesan T. 2013. *In vitro* pollen germination of some wild species of pigeonpea (*Cajanus cajan*) using PGM droplet technique. Indian J. Genet., **73**(2): 211-215.

Jayaprakash P., Annapoorani S., Vikas V. K., Sivasamy M., Kumar1 J., Saravannan K., Punniakotti E. and Sheeba D. 2015. An improved *in vitro* germination medium for recalcitrant bread wheat pollen (*Triticum aestivum* L.). Indian J. Genet., **75**(4): 446-452.

Kakani V. G., Reddy K. R., Koti S., Wallace T. P., Prasad P. V. V., Reddy V. R. and Zhao D. 2005. Differences in *in vitro* pollen germination and pollen tube growth of

cotton cultivars in response to high temperature. Ann. Bot., **96**: 59-67.

Lu Y., Wei L. and Wang T. 2015. Methods to isolate a large amount of generative cells, sperm cells and vegetative nuclei from tomato pollen for “omics” analysis. Front. Plant Sci., **6**: 391.

Mirzaghaderi G. 2010. A simple metaphase chromosome preparation from meristematic root tip cells of wheat for karyotyping or *in situ* hybridization. Afr. J. Biotechnol., **9**: 314-318.

Nadeem M., Younis A., Riaz A. and Ki-Byung Lim. 2015. Crossability among modern roses and heterosis of quantitative and qualitative traits in hybrids. Hort., Environ. Biotechnol., **56**(4): 487-497.

Nguyen V., Delphine F., Andy T., Hamid L., Matthew H., Diane M. and Takashi O. 2015. Addition of rye chromosome 4R to wheat increases anther length and pollen grain number. Theor. Appl. Genet., **128**(5): 953-964.

Rodriguez-Enriquez M. J., Mehdi S., Dickinson H. G. and Grant-Downton R. T. 2011. A novel method for efficient *in vitro* germination and tubegrowth of *Arabidopsis thaliana* pollen. New Phytologist., **197**: 668-679.

Shivanna K. R. and Heslop Harrison J. 1981. Membrane state and pollen viability. Ann. Bot., **47**: 759-770.

Shivanna K. R. and Johri B. M. 1985. The angiosperm pollen: structure and function. Wiley Eastern, New Delhi.

Shivanna K. R. and Rangaswamy N. S. 1992. Test for pollen viability. *In*: Pollen Biology, a laboratory Manual. Narosa, New Delhi, **33**.

Young W. L., Wilen R. and Bonham-Smith P. C. 2004. High temperature stress of *Brassica napus* during flowering reduces micro- and megagametophyte fertility, induces fruit abortion and disrupts seed production. J. Exper. Bot., **55**(396): 485-495.

Zhou L., Lan W., Chen B., Fang W. and Luan S. A. 2015. Calcium sensor-regulated protein kinase, calcineurin b-like protein-interacting protein kinase19, is required for pollen tube growth and polarity. Plant Physiol., **167**(4): 1351-1360.