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Pollen viability and *in vitro* pollen germination studies in *Momordica* species and their intra and interspecific hybrids

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Abstract
The present work aimed to compare five staining techniques for pollen viability and three *in vitro* pollen germination media in *Momordica spp* and their interspecific hybrids. The freshly opened male flower were collected (7.30am to 8.00am) and evaluated for pollen viability and *in vitro* pollen germination. For pollen viability studies five different stains were used *viz*., Tetrazolium stain, I_2KI stain, Aceto-carmine stain, Aniline blue stain and Modified Alexander stain. *In vitro* pollen germination were estimated using three pollen germination solutions *viz*., 10% sucrose, 10% Sucrose + 100 ppm Boric acid and sucrose @15 % + Boric acid @0.25% + Calcium nitrite @300mg solution. Off the pollen viability stains, Modified Alexander stain differentiate viable and non-viable pollen more accurately than other stains. Among the *in vitro* pollen germination media, sucrose @15 % + Boric acid @0.25% + Calcium nitrite @300mg solution showed maximum per cent of pollen germination and pollen tube growth. All five staining methods overestimated the pollen viability.

Keywords: *Momordica charantia*, *M. c.* var *muricata*, *M. balsamina*, Tetrazolium stain, I_2KI stain, Aceto-carmine stain, Aniline blue stain and Modified Alexander stain, In *vitro pollen* germination

Introduction
Quality of pollen are crucial for pollen storage studies, pollen-pistil interaction studies, understanding incompatibility and fertility studies, for breeding and crop improvement and for seed industry (Shivanna and Rangaswamy., 1992) [29]. Pollen viability and vigor decide the quality of pollen (Ottaviano and Mulcahy., 1989) [18]. Pollen fertility and viability have a paramount importance in hybridization programme. Successful pollination is a prerequisite for fertilization and seed set in most plants, and the insight knowledge on pollen biology, including pollen viability, pollen germination and pollen tube growth, is required for any rational approach to increase productivity (Bolat and Pirlak, 1999; Shivanna, 2003) [6, 27]. To differentiate aborted and non-aborted pollens, a simple and hasty technique is required for high throughput. An unambiguous procedure involves pollen deposition on receptive stigma followed by evaluation of seed set. These methods, however, are indirect and sluggish methods, associated with physical and physiological features of pollen with its ability to fertilize the ovule, are universally preferred (Rodriguez-Riano & Dafni 2000) [7, 22]. Pollen germination assays frequently require optimization as well as being time consuming and difficult to reproduce (Boavida, 2007) [5].

Reproduction is a critical step in the life cycle of all organisms and pollination is one of the decisive stage (Piechowski *et al*., 2009; Jha and Dick, 2010) [20, 13] of reproduction process. The male gamete, pollen is surrounded by sporoderm, which consists of the inner layer intine (which is composed of pectin, cellulose and protein) and outer layer exine (synthesized and secreted by the tapetal tissue of the anther and is composed mainly by sporopol-protein). The glycoprotein present on the pistil generally interact with pollen coat protein (Zhang *et al*., 2008) [32]. During the pollination process pollen grain deposited on and adhere to the stigma, the pollen hydrates and germinate by emitting pollen tube, which penetrate the cuticle of the stigma cells and grows through the extracellular matrix of the style. The process culminates in the discharge of male gametes into the embryo sac. In bitter gourd, the pollen tube penetrate papillae tissue within 1 hour of pollination and reaches to the ovule about 6 hour after pollination and fertilization is completed within 18- 24 hours after pollination.

In spite of taking proper care during pollination, breeder fail to get fertile seed during artificial pollination. Unless sterility is the main cause the failure of seed setting may be the result of slow growth of the pollen tube or its early degeneration in the style. To overcome these difficulties it is necessary to study the pollen viability, physiology of pollen germination and pollen tube growth.

There are numerous techniques are available to study pollen viability and the method choice depends on crop (species) and on establishing relationship between test and the fertility (Hanna and Towill 1995) [10]. The most accurate method of pollen viability is the ability of pollen to effect fertilization and seed set (Smith-Huerta and Vasek, 1984; Shivanna and Joshi 1989) [30]. There are different methods to evaluate pollen viability; some noteworthy tests are dye and *in vitro* germination. The dye test have advantages as indicator of pollen viability because they are faster and easier compared with pollen germination, but they do tend to overestimate the viability and real germination of pollen grains. On the other hand, in vitro pollen germination depends on the genotype, environmental conditions, pollen maturity, composition and pH of the medium; thus it is necessary to determine optimum conditions for pollen germination. It is recommended simultaneously use several tests to reflect pollen performance.

The literatures on different tests of pollen viability was reviewed by Dafni and Firmage (2000) [7] and they explained the advantage and disadvantages of each test. The ability to use chemical staining to discriminate aborted from non-aborted pollen grains has well-known practical applications in agriculture. Pollen staining method some time not reliable for most of the species (Barrow, 1981) [4], hence *in vitro* pollen germination is more practical used to determine the pollen viability. The ability of pollen germination play an important role not only on fruit set but also the flower-flower and flower-pollinator interaction. Pollen germination and pollen tube growth very crucial for fertilization and seed development. Due to involvement of the pistillate tissue in the nature, physiological and biochemical investigations on pollen germination and pollen tube growth *in vivo* are rather difficult. Hence *in vitro* germination techniques generally been used extensively on a variety of pollen systems. Such studies have provided considerable information on the physiology and biochemistry of pollen germination and pollen tube growth (Shivana and Johri 1989; Heslop-Harrison 1987; Steer and Steer 1989) [28, 12].

To reach pollen tube to micropyle region, it passes through four stages, a) imbibition phage, b) Lag phage, c) tube initiation phage and d) rapid tube elongation phage. Pollen tube growth proceeds through tip extension and can be affected by many factors, including temperature, medium osmolarity and the availability of sucrose, calcium, zinc and boron (Sawidis and Reiss, 1995; Tylor and Hepler, 1997.) [25, 23].

Momordica genus is one of the major cucurbitaceous vegetable cum medicinal crop and it is grown in most of the Asian countries. The genus comprises 57 species, of which 12 species are distributed in Asian continents (Schaefer and Renner 2010); Out of 12, nine species are presently found in India include cultivated, semi-cultivated and wild species. *Momordica charantia var charantia, M. c. var muricata and M. balsamina* are predominantly grown monoecious species in India. The pollen viability of *Momordica* species lost very rapidly (Desai and Musmade 1998) [8] and till the date very limited systematic studies has been carried out in *Momordica* on pollen physiology studies. Hence, the present investigation carried out to compare different staining method to study pollen viability and i*n-vitro* pollen germination studies in these three species of *Momordica.*

Materials and Methods

Study species

The three species of *Momordica i.e. Momordica charantia, M. c. var muricata and M. balsamina* were used for pollen viability and pollen germination test. These three species are grouped in to gene pool I (Bharathi *et al*., 2012) [3] and they are highly cross pollinated due to their monoecious sex form where male and female flower borne on same plant but at different node position. The inflorescence is solitary and male flower nectary is closed in *M. charantia* whereas, partially closed by corolla scale in *M. balsamina*. The anthesis occurs between 3.30 and 7.30 a.m., when flowers are completely open (Miniraj *et al*., 1993) [17]. The pollen viability is lost relatively rapidly (Desai and Musmade 1998) [8]. The stigma is receptive for 24 hours before and after flower opening, after which it dries and turns brown (Rasco and Castillo, 1990) [21]. The sex ratio (staminate to pistilate flower) in monoecious line throughout the flowering period is typically 50:1 (Rasco and Castillo, 1990) [21] but ratio can vary from 9:1 to 48: 1 (Dey *et al*., 2005) [9]. The ovary of bitter gourd is inferior and three carpels with 14 to 18 ovules (Pillai *et al.*, 1978). In India, Bees are important pollinator vectors (Behera *et al*., 2004) [2].

Study site and plant materials

Seeds of *M. charantia* (DBGS-2), M.c. var *muricata* (DBGS-34) and *M. balsamina* were procured from division of Vegetable Science, Indian Agricultural Research Institute, New Delhi. The seeds were sown in portrays in the month of January-2016 in portrays with media composition of coco peat and vermicompost (1:1), seedling were transplanted at 2-3 leaf stage during spring-summer (April-July, 2016-17) and grown under the insect proof net house condition (Temperature-35-37^{0}C, RH 60-70%). Freshly opened male flowers were used for pollen viability and *in vitro* pollen germination studies.

Pollen viability tests

The literature was reviewed to determine the types of tests used in the past for pollen viability and to determine what problems had been reported for these tests (Dafni and Firmage, 2000) [7] for a list of tests reviewed and the advantages and disadvantages of each test. Base on the literature and facility availability at laboratory condition, we choose five staining methods for our pollen viability experiment.

Tetrazolium Test: Tetrazolium (2, 3, 5 triphenyl tetrazolium chloride) test, commonly known as the TZ test. All living tissues, which respire, are capable of reducing a colour less chemical 2, 3, 5 triphenyl tetrazolium chloride) into a red colored compound formazan by H transfer reactions catalysed by the enzyme dehydrogenases (Norton, 1966). This method provide accurate results for many taxa (Shivanna and Johri, 1989) [28]. However this method has some drawbacks like it tends to overestimate viability (Sedgley and Harbard, 1993) [26], sometime it stains non-viable pollen (Khatum and Flowers 1995) [14]. The concentration of tetrazolium salt, temperature and period of incubation needs to be standardized to get optimal results in various pollen samples (Shivanna and Johri 1989) [28].

Procedure: 1% TTC was prepared by adding 0.2 g. TTC and 12 g sucrose dissolved in 20 mL distilled water. Two drop of the mixture was dropped on a microscope slide and the pollen were dusted over it and covered with a coverslip. Pollen viability counts were made after 30-40 min incubation at 40^0 C under dark place. Pollen grains stained that orange or bright red color were counted as viable.

Iodine- potassium iodide Test (I_2KI): The technique described indicates viability and starch content of pollen-grains. Iodine broke up in a watery arrangement of potassium iodide the tri-iodide-anion edifices with starch, creating blue-black color.

Procedure: Dissolve 1 g potassium iodide and 0.5 g iodine in distilled water to make a final volume of 100 ml. Put 1 or 2 drops of the dye over pollen and mix thoroughly. Place a cover slip and after 5-10min count the number of darkly stained (viable) pollen grains under the microscope.

Aceto-carmine test (2%): Carmine show the presence of cytoplasm. The pollen nucleus is rich in chromatin material and viable pollen stains pink to deep red with aceto-carmine, while sterile (mostly shriveled) pollen does not take any stain and thus remains almost white and transparent (McKellar and Quesenberry, 1992; Marutani, *et al.*, 1993) [16, 15]

Procedure: Weigh 2 g of carmine powder, dissolve it in 95ml of glacial acetic. Add distilled water to make a total of 100ml solution. Boil it, cool and filtered and stored in a refrigerator. Two to three drop of stain was placed on slide and pollen grains were dusted on it followed by covered with coverslip and pollen viability was recorded after 5-10 min. The dark red colored grains are counted as viable pollens.

Aniline blue (Cotton blue) test: Aniline blue stain detect the callose in the pollen walls and pollen tubes (Hauser and Morrison, 1964) [11]. The solution is prepared by adding 200mg/lit of aniline blue in a mixture of 10ml each of phenol, lactic acid, glycerol and distilled water. The viable pollen stained dark blue color, while dead pollen are unstained.

Modified Alexander method: It is a modification of Alexander's stain (Alexander, 1969) [1], which clearly differentiate aborted and non-aborted pollen. Acid fuchsin, present in this dye, stains the protoplasm and malachite green stains cellulose in walls of pollen; dark purple pollen was scored as viable and green as aborted. The non-aborted pollen grains stained magenta-red and aborted pollen grains stained blue-green (Peterson *et al*., 2010) [19].

Protocol: The stain is prepared by adding following constituents in the order given. 10ml of 95% ethanol, 1 ml of Malachite green (1% solution in 95% alcohol), 50 mL Distilled water, 25 mL Glycerol, 5 mL Acid fuchsin (1% solution in water), 0.5 mL Orange G (1% solution in water), 4 mL Glacial acetic acid and Add distilled water (4.5 mL) to a total of 100 ml. Flower bud are fixed in 2 hour before Carnoy's fixative (6 alcohol:3 chloroform:1 acetic acid) before anthesis which sufficiently removes oils and sticky materials and clear the pollen and anther walls. The flower buds or pollen grains are dried and apply 2-4 drops of stain, once the sample is stained, slowly heat the slide over an alcohol burner until the stain solution is near boiling (~30 seconds). A more moderate rate of heating allows better penetration of the dye into the cellulose and protoplasm of the pollen. Slides are covered with cover slip and images are captured under light microscope. The per cent pollen viability was calculated using formula

$$\text{Pollen Viability (\%)} = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains on slide}} \times 100$$

***In vitro* pollen germination test**: *In vitro* pollen germination test were carried out using hanging drop method (Shivanna and Rangaswamy, 1992) [29] with three different germination media suggested by Saoji, (1975) [24] in bitter gourd with media composition of 10 per cent sucrose with 100 ppm boric acid, Kielkowska and Havey (2012) media suggested for cucumber with media composition of 15 % sucrose, 0.025% boric acid, 300 mg of Calcium Nitrate and and a control treatment with 10 per cent sucrose. The culture was maintained in a humidity chamber to prevent evaporation. The freshly opened male flower are collected between 7.30-8.00 a.m. and used for pollen viability and in *vitro pollen* germination studies. Germination of pollen from open male flowers was counted after incubation for 24 h at 25^0C on germination medium. Per cent pollen germination was computed using formula given bellow.

$$\text{Pollen Germination (\%)} = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains on slide}} \times 100$$

Results

Pollen viability using different staining techniques

According to the analysis of variance, all the test showed the significance difference at 1% level of significance except treatment I_2KI, whereas, at 5 % level of significance all the treatment showed significance different (Table 1). Tetrazolium staining method (Fig 1)showed highest number of stained pollen in *M. c*. var *charantia* × *M. c*. var *muricata* (95.33 %) followed by *M. charantia* (94.31%) and lowest was recorded in *M. charantia* × *M. balsamina* (89.66%). In the I_2KI staining (Fig 2) method, non-significant difference was observed among the species and crosses at 1 per cent level of significance. However, it had shown significant value at 5 % level of significance. Among the species and their crosses, the aceto-carmine staining method (Fig 3) showed significance difference at both 1% and 5 % level of significance. *M. charantia* showed highest number of stained pollen (96.42 %) followed by *M. c.* var *muricata* (93.66 %), while *M. charantia* × *M. balsamina* showed lowest per cent of pollen stainability (90.21%). Aniline blue staining method (Fig 4) also showed significant difference among the *Momordica* species and their crosses at both 1 % and 5 % level of significance. The highest per cent of pollen were stained in *M. charantia* × *M. balsamina* (94.00 %) followed by *M. balsamina* (92.71%) and lowest was recorded in *M. c*. var *muricata* × *M. c*. var *charantia* (85.33 %). In modified alexander staining (Fig 5) method, highest percent of pollen viability was recorded in *M. charantia* (86.00 %) followed by *M. balsamina* (84.53 %) and lowest was recorded in *M. charantia* × *M. balsamina* (77.33 %).

Table 1: Pollen viability test in *Momordica* species and its intra and interspecific hybrids using different staining methods.

Treatment	Tetrazolium stain	I_2KI stain	Aceto-carmine stain	Aniline blue	Alexander stain
Population	**Pollen viability (%)**	**Pollen viability (%)**	**Pollen viability (%)**	**Pollen viability (%)**	**Pollen viability (%)**
M M. c. var charantia	94.31±0.88	94.19±0.82	96.42±1.20	91.41±0.87	86.00±0.57
M. c. var muricata	92.42±0.31	92.32±0.34	93.66±0.34	94.00±1.52	84.00±1.52
M. balsamina	91.10±0.56	91.00±0.61	93.34±0.81	92.71±0.68	84.53±0.33
M. charantia ×M. balsamina	89.66±0.33	89.68±0.31	90.21±0.64	87.14±0.33	77.33±0.91
M. charantia × M. c. var muricata	95.33±0.28	93.38±0.42	93.00±0.36	87.66±0.19	82.41±0.88
M. c. var muricata × M. c. var charantia	92.00±0.52	92.00±0.18	91.00±0.48	85.33±1.45	81.00±1.15
CD @1%	2.65	NS	3.65	3.63	4.72
CD @ 5%	1.87	2.43	2.57	2.55	3.32
SE(m)±	0.54	0.54	0.79	1.77	1.01
C.V.(%)	1.02	1.02	1.49	1.56	2.15

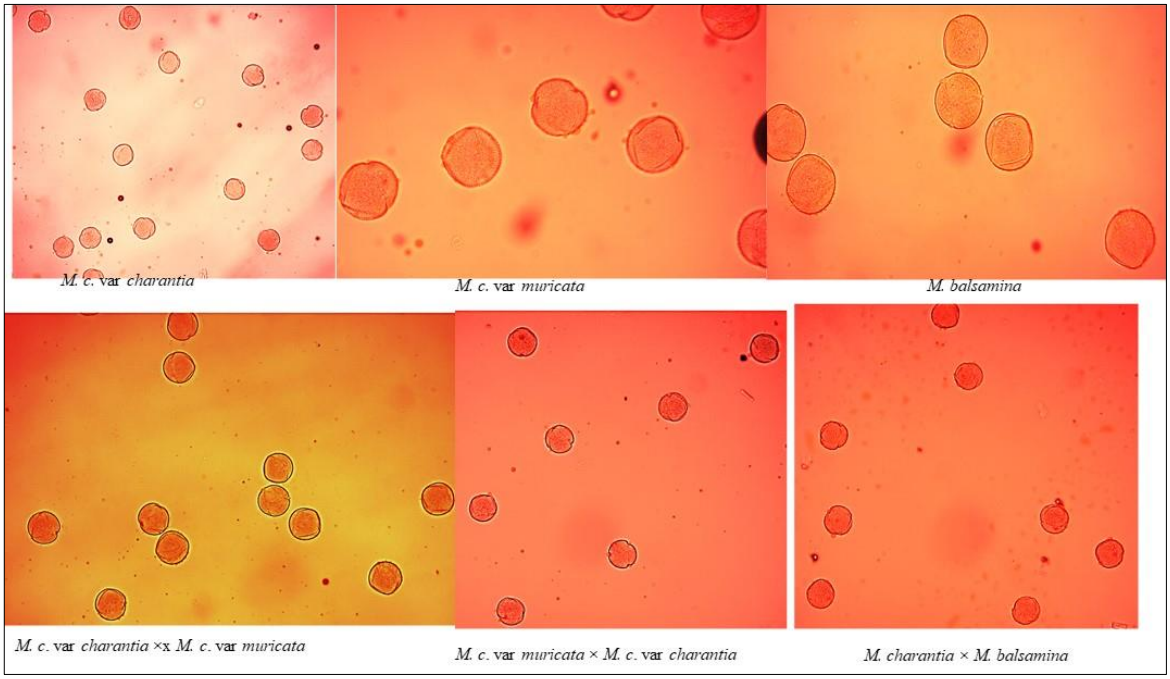

Fig 1: Tetrazolium staing methods in *Momordica species* and inte intra and interspecific hybrids

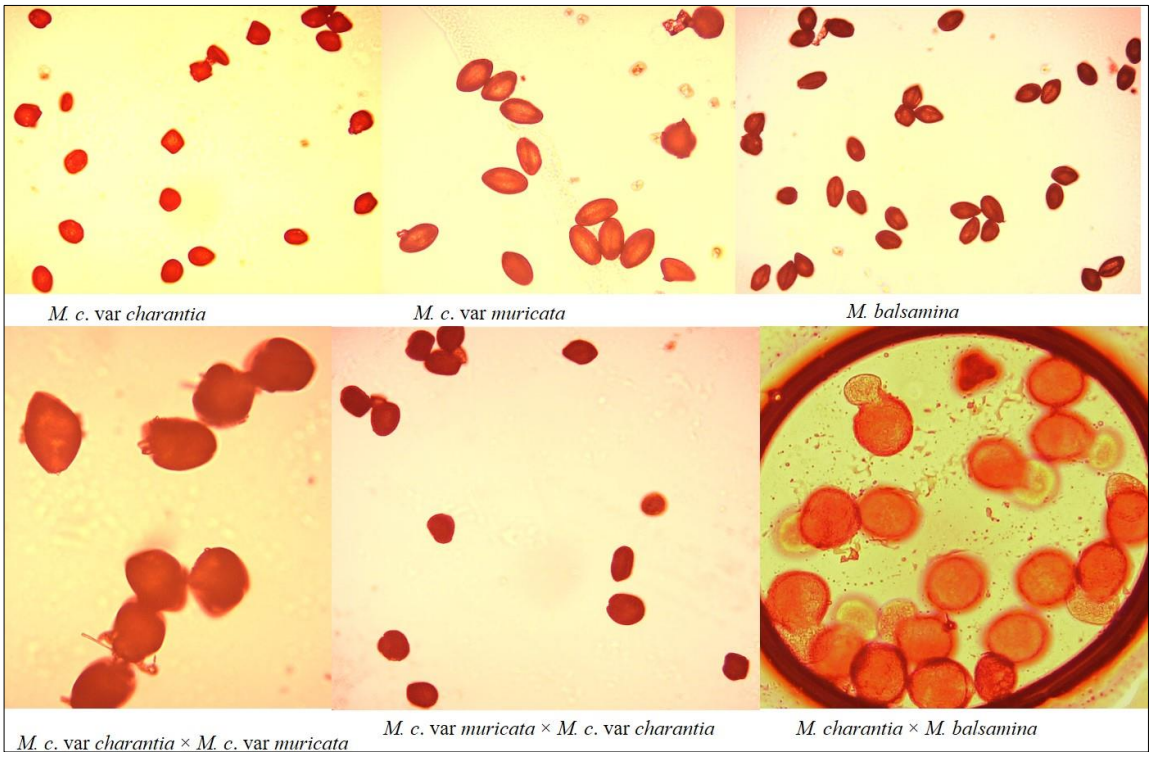

Fig 2: I2KI Staining method for pollen viability in *Momordica species* and its intra and interspecific hybrids

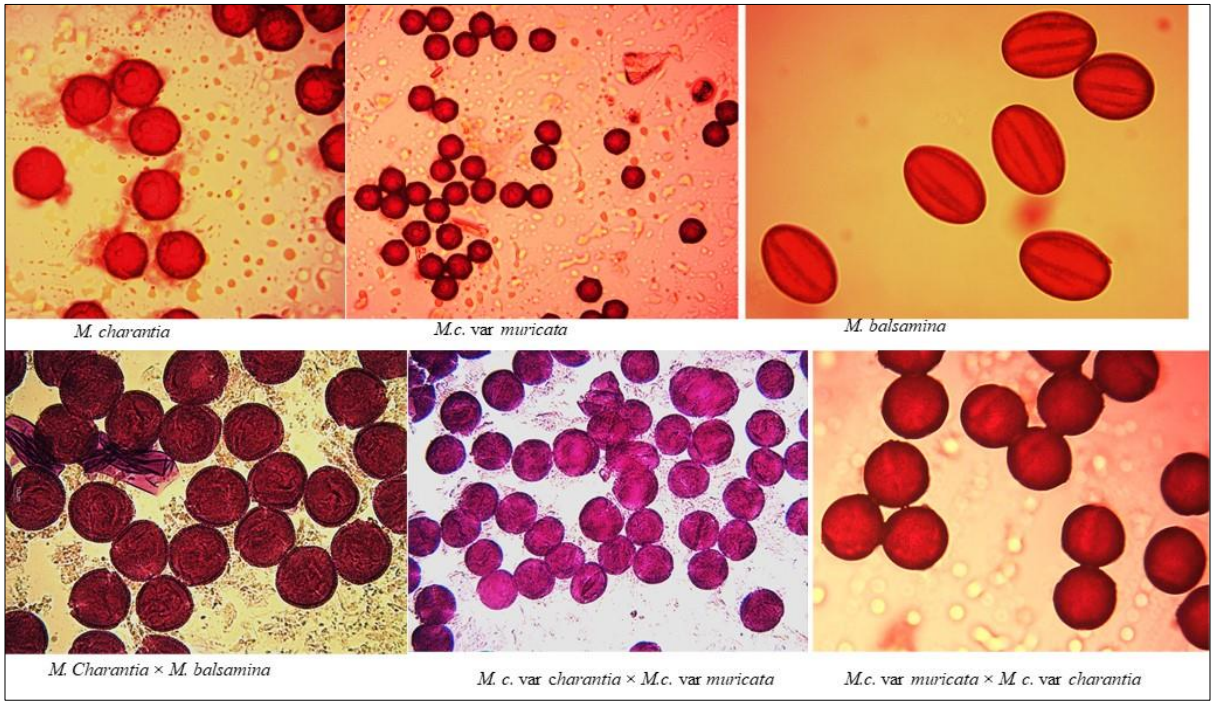

Fig 4: Aceto-carmine staing methods in Momordica species and inte intra and interspecific hybrids

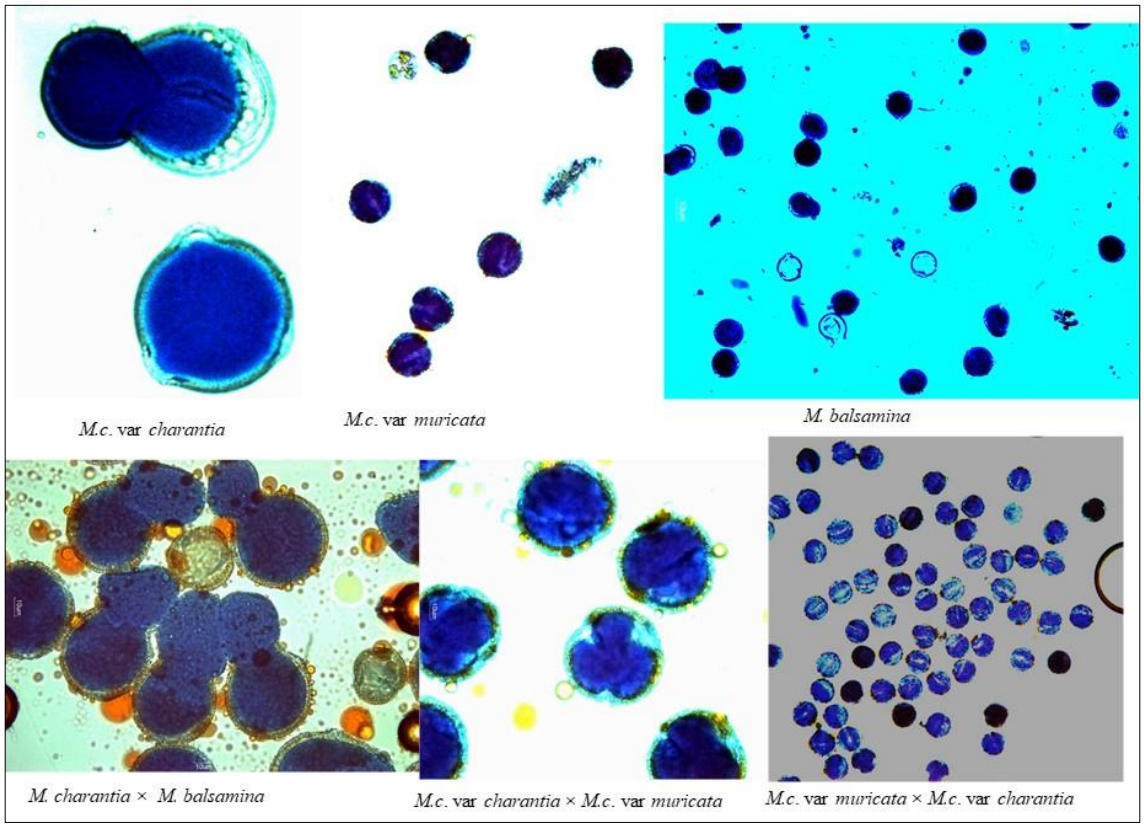

Fig 4: Analine blue staing method in Momordica species and inte intra and interspecific hybrids

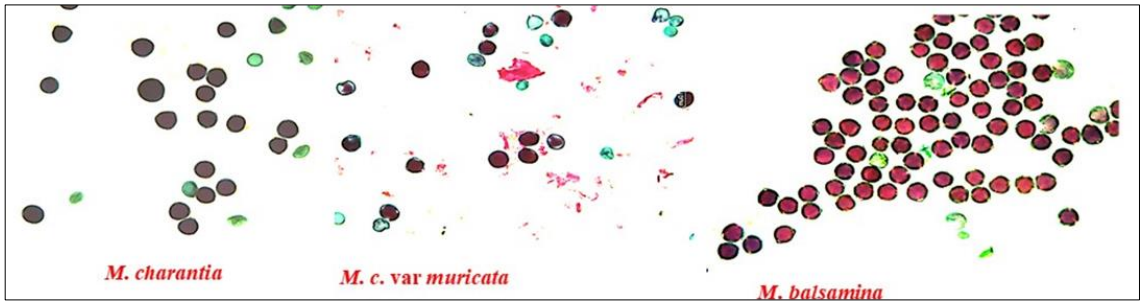

Fig 5: Modified Alexander staing method in Momordica species and inte intra and interspecific hybrids

***In-vitro* pollen germination studies**

Three different composition of pollen germination media was used to study the effect media on pollen germination in *Momordica* species and their intra and inter specific hybrids and the results was presented in Table 2. A germination media with 10 % sucrose showed that pollen germination was less than 50 per cent (Fig 6), where as 44.25 % to 55.00% pollen germination was observed in germinated media with 10 % sucrose + 100 ppm boric acid (Fig 7). *In vitro* pollen germination media with 15 % sucrose + 0.25% boric acid + 300 mg of calcium nitrate showed pollen germination ranged from 62.50 % to 66.00 % per cent (Fig 8). These three medium showed significant difference at 1% and 5 % level of significance.

Among the *Momordica* species and their intra and interspecific hybrids, significance difference for pollen germination was observed only in sucrose 10 % media composition, whereas other two media composition did not shown any significance difference. The germination media with 15 % sucrose + 0.25 % boric acid + 300mg of calcium nitrite showed higher percent pollen germination followed by media with sucrose 10 % + 100 ppm boric acid, where as germination media with 10 % sucrose gave poor response. Among the three species of *Momordica* and their intra and interspecific hybrids, significance difference was observed in media with 10% sucrose whereas, in other two media did not showed significance difference.

Table 2: In vitro Pollen germination of Momordica species and their intra and interspecific hybrids using different *in vitro* media.

Treatments	*M. c.* var *charantia*	*M. c.* var *muricata*	*M. balsamina*	*M. charantia* × *M. balsamina*	*M. charantia* × *M. c.* var *muricata*	*M. c.* var *muricata* × *M. c.* var *charantia*	CD @1%	CD @ 5%	C.V. (%)
	Pollen germination (%)	Pollen germination (%)	Pollen germination (%)	Pollen germination (%)	Pollen germination (%)	Pollen germination (%)			
Sucrose@ 10%	41.50±0.87	40.25±0.95	40.75±1.10	36.50±0.65	35.00±0.71	35.25±0.85	3.53	2.58	4.54
Sucrose@ 10% + Boric acid@100 ppm	55.00±1.29	54.50±1.56	52.75±1.49	44.25±0.48	51.25±1.25	48.25±2.17	NS	NS	5.88
Sucrose @ 15%+Boric acid@ 0.25%+ calcium nitrite@300mg	66.00±0.41	64.75±0.85	65±0.91	62.75±1.65	64.50±0.65	63.00±0.82	NS	NS	2.87
CD @1%	4.09	7.22	5.71	4.88	4.88	5.00			
CD @ 5%	2.71	4.76	3.77	3.22	3.22	3.30			
SE(m)±	0.93	1.16	1.20	1.06	0.91	1.43			
C.V.(%)	2.89	4.37	4.13	3.89	3.68	3.91			

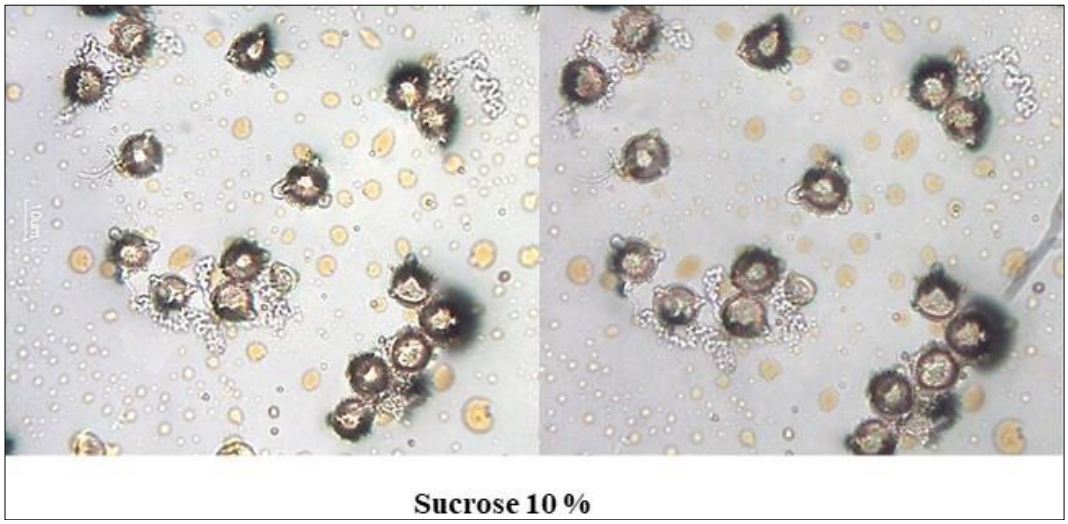

Fig 6: *In vito* pollen germination using 10% sucrose solution.

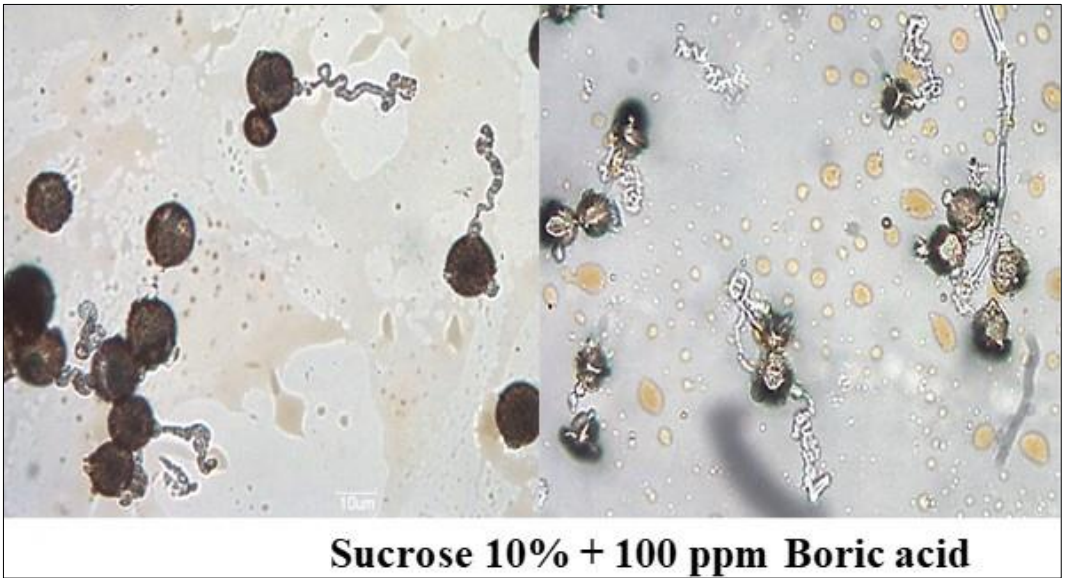

Fig 7: *In vito* pollen germination test using 10% sucrose + 100ppm Boric acid.

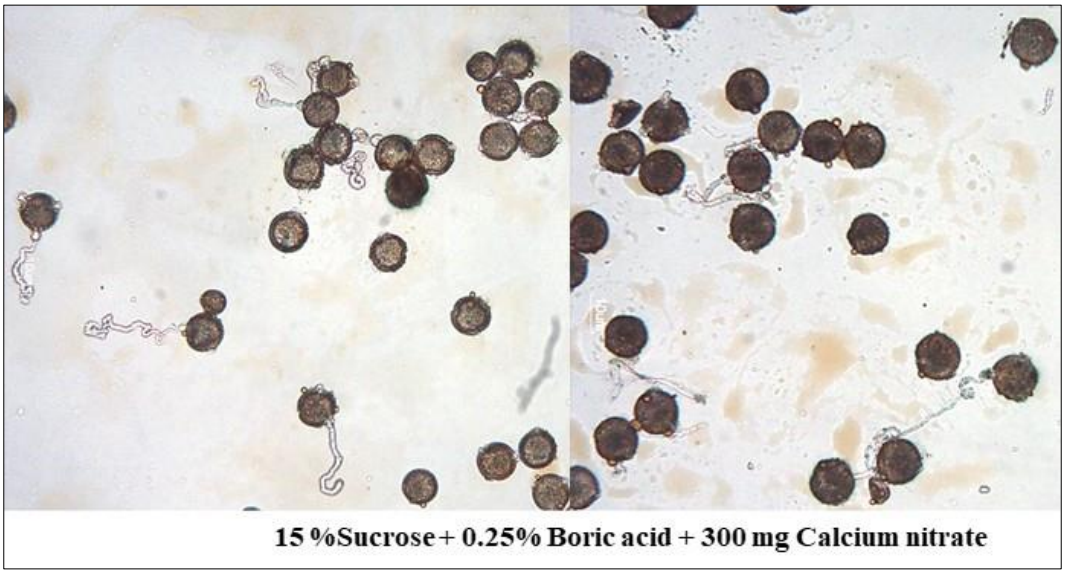

Fig 8: *In vitro* pollen germination test using 15% sucrose+ 0.25% boric acid+300mg Calcium nitrite solution

Correlation between staining methods and *in-vitro* germination media

The person's correlation coefficient was performed among the species of *Momordica* and their intra and interspecific hybrids and between the different sating techniques and germination media used (Table 3). The person's correlation results showed positive and highly significance correlation between modified Alexander stain and Sucrose @ 10% + 100 ppm Boric acid media (0.974), sucrose @ 15 % + Boric acid @0.25% + calcium nitrate @ 300mg (0.871). Aniline blue stain had positive and significant correlation with all three different composition of germination media i.e. with sucrose @ 10% (0.893), Sucrose @10%+100ppm boric acid (0.790) and sucrose @15 % + Boric acid @0.25% +Calcium nitrite @300mg (0.753). The Aceto-carmine stain had positive correlation with Sucrose @10%+100ppm boric acid (0.907) and sucrose @15 % + Boric acid @0.25% +Calcium nitrite @300mg (0.977) these results indicated that the pollen viability using these staining techniques are more reliable than other stain which were used in the present study.

Table 3: Pearson's correlation coefficient for different pollen viability stains and pollen germination test in *Momordica* species and their intra and interspecific hybrids.

Treatments	1	2	3	4	5	6	7	8
Tetrazolium stain (1)	1	0.067	-0.322	-0.531	-0.479	-0.718	-0.391	-0.381
I_2KI stain (2)		1	0.617	0.803*	0.609	0.535	0.711	0.673
Aceto-carmine (3)			1	0.68	0.823*	0.752	0.907**	0.977**
Aniline Blue (4)				1	0.744*	0.893**	0.790*	0.753*
Alexander stain (5)					1	0.659	0.974**	0.871*
Sucrose@10 (6)						1	0.720	0.773*
Sucrose @10%+Boric acid @100ppm (7)							1	0.928**
Sucrose@15%+ Boric acid@0.25%+300mg calcium nitrite (8)								1

Discussion

In the present study among the five staining methods, modified alexander methods could easily differentiate the viable and non-viable pollen grains (Fig 5). In modified alexander staining method viable pollen grains, stained magenta-red stain and non-viable pollen grain stained blue-green (Peterson, *et al*., 2010) [19]. In other methods of staining used in the present study, could not differentiate easily between viable and non-viable pollens. In most of the staining methods, the per cent of unstained pollen grains were very few which indicate very high per cent pollen viability but when the germination test was performed the pollen germination was not showed more than 70 %, this observation indicated that the stains used for pollen viability were could not differentiate between viable and non-viable pollen grains. Barrow (1981) [4] suggested that some time staining techniques could not discriminate between aborted and non-aborted pollen grains, hence *in vitro* pollen germination study has to follow. In bitter gourd, Saoji (1975) [24] reported *in vitro* pollen germination media with sucrose @10 % was best for pollen germination studies, however in or study sucrose @15 % + Boric acid @0.25% + Calcium nitrite @300mg found to be superior. Off the staining methods tested, none was very satisfactory as accusatory predictor of the In vitro pollen germination studied. The pollen germination results are not accurately resemble to pollen viability tested with different method, hence different staining methods could be useful for estimation of pollen viability accomplished with in vitro pollen germination. Similar observation was reported by Sedgle *et al*., (1993) [26] in *Acacia*.

Conclusion

Modified Alexander stain was more efficient for discrimination of aborted and non-aborted pollen in *Momordica* species followed by Aniline blue stain and Aceto-carmine stain. Pollen of Momordica species showed maximum pollen germination and growth in sucrose @15 % + Boric acid @0.25% + Calcium nitrite @300mg solution. Pollen tube growth was abnormal in 10%s sucrose solution.

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