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Pollen Germination and Morphology of Black Pepper (*Piper nigrum* L.)

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ABSTRACT: The pollen morphology and germination of black pepper *var.* Panniyur 1 was studied using Stereomicroscopic and Scanning Electron Microscopic (SEM). The number of anthers in each flower was four and was uniform in all fifty flowers. Anther dehiscence occurred from 11 am and continued till 4 pm and was maximum at 2-3 pm. The number of pollen grains per inflorescence varied from 5,01,500 - 7,00,000. The pollen grains are monosulcate with the mean polar diameter of 10.414 µm and the mean equatorial diameter of 6.309 µm having an exine thickness of 924.8 nm. Acetocarmine test revealed that 91 per cent of the pollen were fertile. The pollen viability percentage by TTC stain and IKI stain was 91.03and 92.4 per cent respectively. *In vitro* germination in Brewbakers- Kwack medium at 5, 10, 20 and 30 per cent sucrose showed highest pollen germination at 5 per cent sucrose.

Keywords: Anther dehiscence, Pollen viability, Pollen fertility, in vitro germination, Black pepper.

INTRODUCTION

Black pepper (*Piper nigrum* L.) one among the distinguished spices known for its pungency and medicinal properties in the family Piperaceae is rightly called the 'King of spices' in view of the legendary position it held in international trade from centuries ago (Kubitzki *et al*., 1993; Sim, 1993; Ravindran, 2006). Wet evergreen forests in the hill ranges of Western Ghats in South India, now declared as one among the "global hot spots of biodiversity" is the primary centre of origin of this valued spice crop (Ravindran, 2006). India is known as the home of spices and Indian spices are most sought after globally, due to their exquisite aroma, texture and taste (Ravindran 2006). Kerala, the southernmost State of India occupies an extensive share of the Western Ghats which is a rich repository of wild relatives of tropical spices, especially of the genus Piper, which occurs among the moist deciduous semi-evergreen and evergreen forests (Ravindran 2000; Sasidharan, 2010). Kerala contributes about 97% of the India's pepper production (Parthasarathy *et al*., 2006). Black pepper is having several medicinal properties such as defensive role against infection by insects, animals and microbes treating digestive disorders, gastric problems, diarrhoea, indigestion and respiratory problems (Shun *et al*., 2007). India produced with 61,000 metric tonnes of black pepper from an area of 1,37, 378 ha in the year 2019-20. Apart from India, pepper is produced in Brazil, Sri Lanka and Vietnam on large scale. Vietnam is the world's largest producer and exporter of pepper (Spices Board, 2019). Kerala, Karnataka and Tamil Nadu are the major black pepper producing states in India. The species and cultivars of Piper examined are difficult to distinguish on the basis of plant morphology which makes development of an infrageneric classification challenging. However, the variation in pollen morphology between varieties offers another avenue for demarcating the taxa (Divya *et al*., 2015).

Piper nigrum exhibits much uniformity in pollen morphology (Ravindran *et al*., 2000). The size and pollen types in Piper was reported (Erdtmann 1952; Walker and Doyle 1975). Chen *et al*., (2018) reported that (Scanning Electron Microscopy) SEM observation showed pollen grain size of black pepper was <10 µm in diameter, categorised under myosotis, spherical shaped, radially symmetrical and with irregular pinulose sculpturing.

Pollen counts are essential feature of research on pollination like estimation of male reproductive output, comparison of functional gender of different plant morphs, quantification of pollen removed by pollinator visits and estimation of airborne pollen levels causing allergy (Kearns and Inouye, 1993). Previous research has proven the unlikeliness of morphological differences among black pepper cultivars pollen

(Ravindran *et al*., 2000). The total amount of pollen per anther varies with the cultivar.

The objectives of this study are to determine the Anther dehiscence time and mode, pollen characteristics, pollen viability and fertility, *in vitro* and *in vivo* germination of black pepper, while in tandem, the aim is also to improve the efficiency of conventional breeding.

MATERIAL AND METHODS

The experiment was be carried out in black pepper variety Panniyur-1 in the field grown pepper plants and bush pepper plants maintained in pots. Twenty-five plants of black pepper variety, Panniyur 1 of uniform age grown in the Instructional Farm, College of Agriculture, Vellayani were selected and marked for the study. The plants were observed from March 2018 to March 2019 for studying the pollen morphology in black pepper. Two-year-old fifty bush pepper plants maintained in the department of Plantation Crops and Spices in pots used for pollen germination in black pepper.

Anther dehiscence time. Fifty inflorescences were selected and tagged from twenty-five plants. The time of an thesis was recorded from the time of anther emergence till it dehisces and loose completely. The data were recorded from 10 am to 4 pm at one hour interval and the dehisced anthers were removed from the spike after each count to avoid recounting.

Anther dehiscence mode. Fifty flowers were selected from fifty inflorescences. Each flower was observed for anther emergence to the dehiscence of anther and the mode of dehiscence was found out.

Pollen characteristics. For pollen morphology studies, the anther at the time of maturity were collected and preserved immediately in 70% ethanol. Slide preparation for pollen morphology studies were made by the acetolysis method proposed by Erdtman (1952) and Nair (1960). The preserved material of anthers was transferred to a centrifuge tube and crushed with a glass rod. The dispersion was sieved through a brass mesh of 48 divisions cm^{-2} and was collected in a glass centrifuge tube. After centrifugation in centrifuge of 2000 rpm for 5 minutes, the supernatant was decanted and the pollen grains after washing in glacial acetic acid were treated with acetolysis, a mixture consisting of acetic anhydride and concentrated sulphuric acid (9:1) in the centrifuge tube. A glass rod was placed in each tube and was transferred to a water bath at 70-100°C for three to five minutes, till the medium became brown in colour. Centrifugation of this mixture was carried out at 2000rpm for five min, the supernatant was decanted off and glacial acetic acid was added to the sediment and again centrifuged and acid was decanted. The permanent slides of acetolysis pollen grains were made by mounting them in glycerin jelly of 2 drops and the edges were sealed with paraffin wax**.** The prepared slide was taken for observation of pollen shape, pollen size, viable and nonviable pollen using Scanning Electron Microscope at Sophisticated Instrumentation and Computation Centre, University of Kerala.

Pollen fertility. Pollen stain ability as an index of fertility was determined by staining pollen grains by acetocarmine glycerin staining technique. A modification of the technique developed by Marks (1954) was used for the study. Matured anthers were collected, kept in clean slide. To this, one drop of acetocarmine was added and macerated to release the pollen grains. Debris were removed and one drop of glycerine was added over this and mixed once again using needle and covered by cover slip. After 10 minutes the slides were examined under stereo microscope. The number of stained and unstained pollen grains were counted. The stained pollen grains were considered as fertile whereas the unstained, undersized, partially stained and shriveled pollen grains were counted as sterile. The pollen fertility was calculated as

$$\text{Pollen fertility percentage} = \frac{\text{Number of fully stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

The mean percentage of fertile pollen was calculated from six samples with each containing about 59 to 85 number of pollen grains.

Pollen viability test

(i) Iodine Potassium iodide test. Potassium iodide (1g) and 0.5g iodine were dissolved in 100 ml distilled water for preparing IKI solution. Pollen grains were placed in IKI solution for10 minutes and viability was assessed by counting the dark brown or red coloured pollen grains (Sulisoglu and Cavusoglu, 2014). The test was carried out in six samples and the pollen viability was calculated as

$$\text{Pollen viability percentage} = \frac{\text{Number of fully stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

(ii) Triphenyl Tetrazolium Chloridetest. One per cent TTC (0.2 g TTC and 12 g sucrose in 20 ml distilled water) was used and a drop of the mixture was dropped on a microscope slide and the pollen was spread with a slim brush and covered with a cover slip. Pollen viability counts were made after 10 min. Pollen grains stained orange or bright red colour were counted as viable (Sulisoglu and Cavusoglu, 2014). The test was carried out in six samples

$$\text{Pollen viability percentage} = \frac{\text{Number of fully stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

Pollen germination tests

(i) *In vitro* pollen germination. *In vitro* pollen germination was conducted to determine the effect of different nutrients like sucrose, boron and calcium nitrate at various concentrations. Different graded of sucrose (5, 10, 20, 30, 40%) were prepared and used individually and also in combination. In addition to this, Brewbaker - Kwack medium (Brewbaker- Kwack, 1963) was also used for *in vitro* germination test. One or two drops of each medium were placed separately on a clean glass slide. Freshly dehisced pollen grains were added into the solution and spread thoroughly. The slides were incubated in petridish lined with moist filter paper for 24h. After incubation, a drop of cotton blue was added to it and allowed to disperse. The pollen grains which have produced pollen tubes longer than the diameter of the pollen were recorded at a magnification of 40X (Belavadi and Ganeshaiah, 2013).

***In vivo* germination.** Ten flower buds from five inflorescences were selected and remaining flower buds were removed. Each inflorescence was covered with polythene cover. Pollen grains were collected from other flowers after anther dehiscence and placed on the stigma of each flower. *In vivo* pollen germination was investigated by observing pollen germination and pollen tube entry into the stigma in these manually pollinated black pepper flowers.

DAB (Diaminobenzidine) test. Pollinated stigma was collected after 6h and kept in moist chamber for 2h. and stained stigmas were placed on a clean slide and added a drop of glycerin. Mounted stigmas were observed under microscope Leica MZ95 and were photomicrographed.

ABF (Aniline Blue Fluorescence) method. Fix the pollinated stigma for 24 hand store the min70% ethanol. Then transfer the fixed stigma to 4N NaOH (delicate material) for clearing. The temperature andthe period of clearing depend on the size and texture of the pistil. It was found that overnight clearing at laboratory temperature was sufficient. The period of clearing can be decreased by increasing the temperature 60 ℃. Transfer the fixed stigma to water in a petridish /beaker and rinse them carefully. The clean stigmas were rinsed in water and 1:1 mixture of aniline blue and glycerine, alternatively. Leave the softened and rinsed stigma in aniline blue overnight and then mount them in a drop of glycerine. Gentle pressure was applied on the cover glass to achieve required degree of spreading of the tissue. Then observed under fluorescent microscope.

RESULTS AND DISCUSSION

Anther dehiscence time and mode

Anther dehiscence occurred from 11 am and continued till 4 pm and was maximum at 2-3 pm. According to Nybe *et al*., (2007) anther dehiscence took place between 14.30- 15.30 hrs (2.30-3.30 pm). Anther dehiscence mode was obtained from fifty flowers of fifty inflorescences taken from twenty-five black pepper plants grown in the field. All black pepper flowers showed longitudinal dehiscence. Stereomicroscopic observation of anther dehiscence opening revealed longitudinal splitting of anther to release the pollen.

Pollen characteristics *Piper* spp. exhibits much uniformity in pollen morphology, except for the variation in size. The pollen grains were small, the diameter along the equatorial axis ranged between 8.0 and 18.0 μm and along the polar axis, between 6.5–15.0 μm. The pollen grains were monosulcate, the sulcus extending up to the lateral extremities. Very rarely the grains are non aperturate or porate (Rahiman, 1981). The sulcus is in the form of a narrow slit with thick borders all along the periphery. The exine surface is reticulate, the bronchi being large and irregular on the distal polar surface. This is supported by Federico *et al*. (2017) in their study on Jatropha cultivars and also Sanja *et al*., (2013) on sweet cherry cultivars study.

The pollen morphology was studied using SEM revealed monosulcate (Fig. 1) pollen grains with the mean polar diameter of pollen grain was10.414 µm and the mean equatorial diameter was 6.309 µm (Fig. 2) having a mean exine thickness of 924.8 nm (Fig. 3) are shown in the Table 1. In *P. nigrum* the pollen grains measure along the equatorial axis from 9.5–13.0 μm, the mean being 11.0 μm; and along the polar axis 7.0–10.5 μm having a mean of 8.84 μm (Rahiman, 1981). The grains are spheroid to suboblate, rarely pyramidal, sulcus measures 7.0–11.0 μm in length and 1.0–2.0 μm in breadth. According to Nybe *et al.*, (2007), the mean diameter of pollen grain was 9.86 µm. Martin and Gregory (1962) reported the mean diameter of pollen grain of black pepper as approximately 10 µm. According to Divya *et al*., (2015) the pollen grains of black pepper was monosulcate. Grains monosulcate. Exine ornamentation verrucate-spinulate with prominent inconspicuous to conspicuous hemispherical hold, and very small column. Exine thickness: 1 µm. Size of pollen: Ac: P: 10-11 µm; E1: 10 µm; E2: 8 µm. UAc: P: 9-10 µm; E1: 8 µm; E2: 6 µm. Expansion due to acetolysis is 2 µm (Divya *et al.*, 2015).

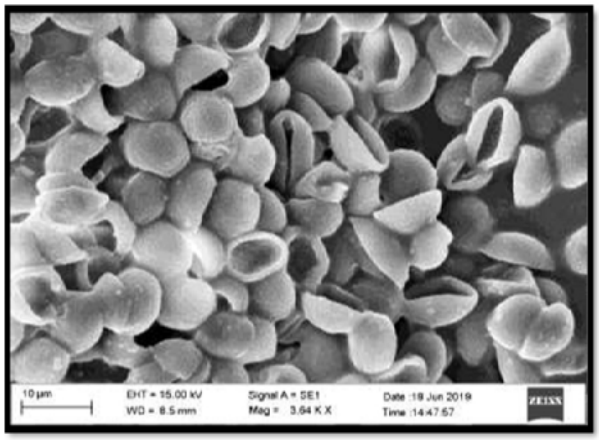


Fig. 1. Scanning Electron Microscopic image of pollen grains of *Piper nigrum* L. (Scale bar 10 µm).

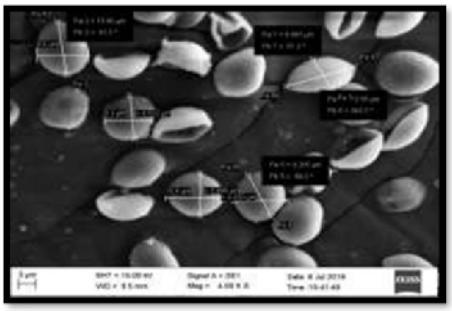


Fig. 2. Equatorial and Polardiameter (Scalebar 3µm).

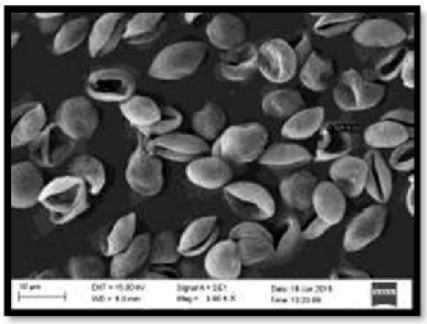


Fig. 3. Exine thickness (Scale bar 10µm).

Table 1: Polar and Equatorial axis diameter of pollen grains of *Piper nigrum* L.

Sr. No.	Polar axis diameter (µm)	Equatorial axis diameter(µm)
1.	10.60	9.0
2.	8.622	8.3
3.	12.55	6.687
4.	10.4	8.496
5.	9.898	6.533
Mean	**10.414**	**6.309**

Pollen fertility. The pollen fertility studies by Acetocarmine test revealed that 91 per cent of the pollen was fertile.

Stereomicroscopic image of pollen fertility by acetocarmine shown in the Fig. 4. Pollen fertility can be assessed by acetocarmine and glycerin staining technique (Radford *et al.*, 1974). The stained pollen grains were treated as fertile and unstained pollens were counted as sterile and presented in Table 2.

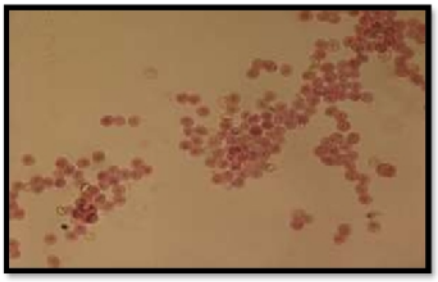


Fig. 4. Pollen fertility test by Acetocarmine in *Piper nigrum* L.

Table 2: Pollen fertility test by Acetocarmine in *Piper nigrum* L.

Anther	Total number of pollen	Number of stained pollen	Fertility Percentage
Replication 1	78	72	92.3
Replication 2	69	61	88.4
Replication 3	85	79	92.9
Replication 4	59	52	88.1
Replication 5	62	57	91.5
Replication 6	70	65	92.8
Mean			**91**

Pollen viability. The pollen viability percentage by TTC stain and IKI stain was 91.03 per cent and 92.4 per cent respectively and represented in Table 3. The stereomicroscopic image of pollen fertility by TTC and IKI is shown in the Fig. 5 and 6. Assessment of pollen viability is a pre-requisite and also important in studies on pollen storage, reproductive biology and hybridization (Harrison *et al.*, 1984). Pollen viability is a critical factor, which is considered as an important parameter of pollen quality (Dafni and Firmage, 2000).

Table 3: Pollen viability test by TTC in *Piper nigrum* L.

Anther	Total number of pollens	Number ofstained pollens	Fertility Percentage
Replication 1	82	75	91.4
Replication 2	67	62	92.5
Replication 3	65	60	92.3
Replication 4	72	65	90.2
Replication 5	51	46	90.1
Replication 6	78	70	89.7
Mean			**91.03**

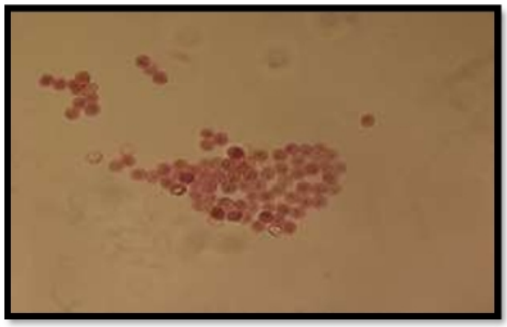
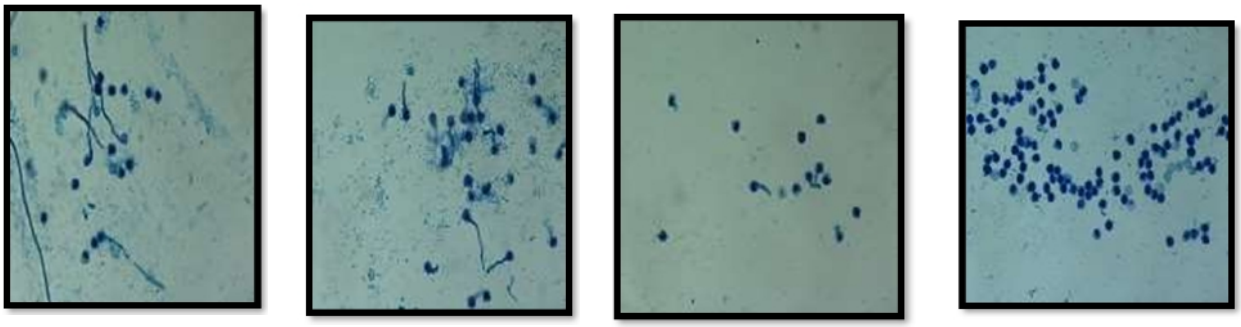


Fig. 5. Pollen viability test by TTC in *Piper nigrum* L.

Fig. 6. Pollen viability test by IKI in *Piper nigrum* L.

Pollen viability

(a) *In vitro* germination. In vitro germination in Brewbakers- Kwack medium at 5, 10, 20 and 30 per cent sucrose showed highest pollen germination at 5 per cent sucrose. The steromicroscopic image of pollen germination is shown in the Fig. 7. *In vitro* pollen germination test indicated that highest percentage of pollen germination and tube elongation was observed in Brewbakers medium. It contains sucrose which acts as a nutritive material for pollen germination (Johri and Vasil, 1961) and helps in maintaining osmotic balance between the germination media and pollen cytoplasm. *In vitro* germination has been extensively used since pollen grains of large number of species readily germinate *in vitro* on a simple reaction with a carbohydrate, source, boron and calcium (Shivanna and Rangaswamy 1992). Chen, (2011) published the germination medium protocol for pollen viability test on *P. colubrinum*, which was then adapted to *P. nigrum* pollen. The optimised liquid medium consisted of 10% sucrose, 100 mg/L boric acid, and 300 mg/L calcium nitrate.



(a) 5% sucrose (b) 10%sucrose (c) 20% sucrose (d) - 30% sucrose

Fig. 7. Pollen germination at different concentration of sucrose (Brewbakers-Kwack medium).

(b) *In vivo* germination. Twenty-five flowers from five inflorescences of twenty-five black pepper plants grown in field were used for observing pollen germination and pollen tube entry into the stigma of manually pollinated flowers. *In vivo* germination was done through DAB test and aniline blue fluorescence method for 6 hours and 24 hours and fluorescent microscopic images were taken and shown in Fig. 8 and 9.

Sucrose is the best carbohydrate source for pollen germination, as it maintains the osmotic pressure of the medium and acts as a substrate for pollen metabolism. The germination percentages were significantly low in higher concentration of sucrose medium and the optimum concentration of sucrose varies from species to species (Shivanna and Johri, 1985). In the present investigation, the pollen tube growth was highest at 10 per cent concentration, whereas, the lower pollen tube growth was observed in higher concentration of sucrose at 20 and 30 per cent.

Pollen germination and growth of pollen tubes are important research materials for physiological, biochemical, biotechnological, ecological, evolutional and molecular biological studies (Ottavio *et al*., 1992). Pollen grain can remain viable even upto 5 days after the opening of the flowers in black pepper (Sasikumar *et al*, 1992). Another study found that pollen collected after 20 hrs showed relatively low germination percentage and short pollen tube elongation. The pollen collected from this stage may be non-viable, even though the pollen is able to achieve satisfactory high germination percentage and pollen tube elongation. According to Harrison (1979), non-viable pollen grains may hydrate to the same extent as living pollen grains, swell, and even develop short tubes before the tubes eventually rupture. In the pollen viability study, results suggest that black pepper pollen were more viable between five and 10 hrs after anther dehiscence (Chen *et al*., 2018).

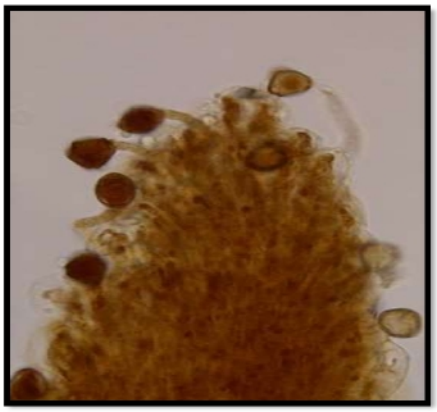


Fig. 8. Pollen viability through *In vivo* by DAB method under Stereomicroscope.

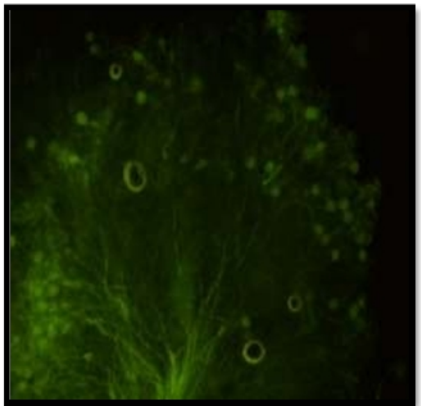


Fig. 9. Pollen viability through *In vivo* by aniline blue fluorescence method.

CONCLUSION

The pollen grain size varies from variety to variety. In the present investigation, the polar and equatorial diameter and exine thickness of pollen grain of Panniyur -1 variety of black pepper were 10.414µm, 6.309µm, 924.8µm respectively. Ninety-one per cent of pollen were identified as fertile using acetocarmine stain. The pollen viability of Paniyur-1 variety was found 91.03 and 92.4 per cent in TTC and IKI methods respectively. The maximum pollen grain germination was observed in *in vitro* at 5 and 10 per cent sucrose concentration. This was further confirmed with the *in vivo* germination tests in the field. The pollen morphology thus deciphered can be helpful in studying the black pepper relationships at the morphological and genetic levels and will also help in rational selection, comparison and improvement of this important agronomic crop.

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Conflict of Interest. None.

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