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Assessment of pollen morphology and hybridization in pomegranate (*Punica granatum*)

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ABSTRACT

Pollen studies act as a prerequisite for choosing a compatible pollen parent and help plant breeders to achieve successful crossing between different cultivars. An experiment was conducted during 2018–19 at Fruit Research Farm of Regional Horticulture Research and Training Station Bajaura, Kullu, Himachal Pradesh to evaluate 8 commercial genetically distant pomegranate (*Punica granatum* L.) cultivars (Chawla, Nabha, P-26, Kandhari Kabuli, G-137, Jodhpur Red, Mridula and Bhagwa) for pollen morphology (shape and size) and viability. Hybridization studies were also carried out to assess the role of cross pollination in fruit set to infuse blood red aril colour and soft seededness in the selected cultivars. The compatibility behaviour was studied by using different cross combinations of cultivars followed by recording their fruit set percentage. It could help breeders understand the performance of cultivars as pollinizers which would ultimately help them in hybridization programs. Pollen viability of the pomegranate germplasm was determined using 2% acetocarmine. Highest pollen viability was recorded in Bhagwa (94.29%) followed by Chawla (91.21%). In hybridization studies, highest fruit set per cent among 16 crosses was observed in Mridula × P-26 (77.80%). The differences in fruit set percentage could be due to varied degree of compatibility between different genotypes, behaviour of pollen with respect to viability percentage, differences in growth rate of pollen tube and might also be due to differences in temperature during bloom period.

Keywords: Cultivars, Hybridization, Pomegranate, Pollen, Viability

Pomegranate (*Punica granatum* L.) is a prime fruit crop that commercially grows in tropics and subtropics of the world. Both edible and non-edible parts possess various metabolites which are source of functional food and nutraceuticals to encourage human health (Kulkarni *et al.* 2004). India is the largest producer of pomegranate in the world with annual production of 3212.32 thousand MT with an export of 99.04 thousand MT fresh fruits amounting to ₹688.77 crores (Anonymous 2022). Its diversification extends through climatic variability in Maharashtra, Karnataka, Rajasthan and Himachal Pradesh states of the country. In Himachal Pradesh, it has 2.77 thousand ha of area under cultivation with productivity of 1.14 MT/ha extending in all districts except Lahaul Spiti and Kinnaur (Anonymous 2019). Flowering time, pollen characters and the morphological features of flowers are useful in the classification of different cultivars (Babu 2010). To meet export market standards, the fruit has to be of a certain size; this is related to pollination success and consequently to seed number. Plant pollen has excellent genetic conservation and is not easily affected by environmental factors (Shangshang *et al.* 2015). Information on pollen characters, viz. pollen viability and pollen morphology are a prerequisite for carrying out a meaningful crop improvement programme. Pollen studies have been widely used in many fruit trees (Du *et al.* 2006, Wetzstein *et al.* 2011, Kumar 2012, Engin and Gokbayrak 2016, Kumar and Kaur 2019, Sharma *et al.* 2021).

However, there is some lack of knowledge about pollen morphology of pomegranate cultivars in different geographical areas of Himachal Pradesh. Thus, pollen viability and morphology of different pomegranate cultivars were reported in this study. The acceptability of a cultivar by the consumers depends largely on its fruit quality traits. With the increase in the base of pomegranate variability, it has become essential to further develop better cultivars through hybridization. Therefore, with a view to infuse blood red aril colour and soft seededness in some of hard seeded commercial cultivars, the present investigations were carried out on eight genetically distant pomegranate cultivars.

MATERIALS AND METHODS

The experiment was conducted at Regional Horticultural Research (RHR) and Training Station (TS) of Dr YS

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Table 1 Pomegranate cultivars used to conduct present study

Cultivar	Origin/cultivation	Salient feature
Chawla	Cultivated mainly in Haryana	Pink red fruits, light pink-coloured arils and hard seeds
P-26	Seedling selections from *Muscat*	Large fruit size, yellowish green rind, red tinge, pinkish white arils, sweet in taste and seeds hard. Superior to Ganesh for yield and fruit weight
Nabha	Mainly grows in Haryana	Medium fruit size, dark pink arils, acidic taste and hard seeds
Kandhari Kabuli	Introduced from Afghanistan	Large blood red fruit, deep red rind, hard seeds, sweet acidic taste
Jodhpur Red	Mainly grows in Rajasthan	Large fruit, orange red colour, medium pink-coloured arils, soft seeds
G-137	Clonal selection from *Ganesh* which grows mainly in Maharashtra	Round and medium red coloured fruits, medium pink-coloured arils, soft seeds, fruit weight up to 400–500 g
Mridula	F_2 selection from the cross Ganesh × *Gul-e-Shah Red*	Deep red, shiny skin, juicy arils deep red in colour, soft seeds, fruit weight up to 250–300 g
Bhagwa	F_2 selection from the cross *Ganesh* × *Gul-e-Shah Red* observed in farmer's field	Fruits have an attractive glossy red rind. Arils are blood red in colour with soft seeds

Parmar University of Horticulture and Forestry at Bajaura (Kullu), Himachal Pradesh, (31°50' N, and 77°09' E, at an elevation of 1230 m amsl) during 2018 and 2019 to evaluate 8 commercial genetically distant pomegranate (*Punica granatum* L.) cultivars (Table 1) for pollen morphology and viability. The study area was typically sub-temperate where the winters are cold and summers are very hot with maximum and minimum temperature of 31.5°C (June) and 0.8°C (January), respectively. July and August are rainy months with an annual rainfall of 150–175 cm.

Pollen collection: For pollen studies, flower buds were collected and immediately stored in Carnoy's solution (absolute ethanol and acetic acid at 4:1) to avoid any physiological damage. After fixing for 48 h, buds were transferred to 70% (v/v) ethanol before being put in a refrigerator (Engin and Gokbayrak 2019). Anthers were slowly detached from the flowers using forceps and arrow headed needles. For hybridization studies, flower buds at balloon stage were collected and anthers were detached and kept on a piece of butter paper under partial shade for dehiscence. Collected pollen grains were stored in glass vials. Freshly dehisced and small samples of pollen grains were drawn out from these vials for cross pollination studies.

Pollen viability (Acetocarmine solution 2% test): For pollen viability studies, flower buds were collected from the plants growing under natural conditions from different localities of selected areas of the RHR and TS Bajaura, Kullu. Fresh acetocarmine solution (2%) was prepared by dissolving 45 ml of glacial acetic acid and 2 g of carmine powder to make the final volume 100 ml (Kumar and Kaur 2019). The mixture was boiled carefully and then filtered with Whatman No. 1 filter paper (pore size=11 μm). Pollen fertility was estimated by mounting mature pollen grains in glycerol-acetocarmine (1:1) mixture. Nearly 500–700 pollen grains were analyzed in each case for evaluating pollen fertility and pollen size. About 10–20 fresh slides were prepared from different anthers/flowers for different individuals of a particular population and then were analyzed according to Completely Randomized Design (CRD) in each case. Well-filled pollen grains with stained nuclei were taken as apparently fertile, while shriveled and unstained pollen grains were counted as sterile. Photomicrographs of pollen grains were made from freshly prepared slides using Biovis image plus Digital Imaging System.

Pollen morphology (size and shape): The length and breadth of pollen grains were measured with a Digital Imaging System microscope. Images were analyzed for the pollen morphology by using Biovis Image plus (software). The shape of pollen grains was observed after staining with acetocarmine solution (2%).

Hybridization studies: Hybridization studies with different cross combinations (16) were done in the Pomegranate orchard at Regional Horticulture and Research and Training Station, Bajaura (Kullu), Himachal Pradesh. Based on their salient features (Table 1), 4 female parents (Mridula, Bhagwa, G-137 and Jodhpur Red) and 4 male parents (Kandhari Kabuli, P-26, Nabha and Chawla) were crossed with different cross combinations. Branches were selected and all the opened flowers and undeveloped buds were removed. Unopened blossoms were emasculated by removing sepals, petals and stamens to prevent self-pollination. The emasculated flower clusters were covered with muslin cloth bags and properly tied and labeled. Pollination of the emasculated buds was done on the day of flowering either with fresh pollen (for cultivars where flowering period coincided) or with stored pollen (for cultivars where flowering periods did not coincide). The pollens were applied to stigmas with camel hair brush followed by bagging. Each twig was labeled to avoid any contamination. The bags were removed after fruit setting.

Per cent fruit set in pollinated flowers was calculated as the ratio of the number of buds which set into fruits to that of the total pollinated buds. Per cent fruit set was calculated as:

$$\text{Fruit set per cent} = \frac{\text{Number of buds which set into fruits}}{\text{Total number of buds pollinated}} \times 100$$

The data obtained from the present investigation were subjected to statistical analysis as per the methods suggested

Table 2 Pollen studies of some pomegranate cultivars

Cultivar	Pollen viability (%)	Pollen shape	Pollen size (µm)
Chawla	91.21 (72.80)	Circular	25.75 × 19.43 - 17.32 × 13.27
Nabha	90.78 (72.41)	Circular	25.32 × 19.09 - 15.70 × 11.52
P-26	90.58 (72.11)	Circular	24.31 × 18.79 - 15.10 × 12.35
Kandhari Kabuli	90.41 (71.98)	Circular	22.24 × 16.56 - 13.36 × 10.41
G-137	70.49 (57.08)	Circular	23.79 × 17.53 - 13.70 × 9.70
Jodhpur Red	89.62 (71.30)	Circular	24.89 × 18.53 - 15.63 × 12.08
Mridula	44.23 (41.66)	Circular	22.75 × 19.79 - 16.89 × 11.90
Bhagwa	94.29 (76.37)	Circular	23.71 × 17.23 - 14.65 × 10.24
CD (P=0.05)	3.40	-	-

*Figures in the parenthesis are angular transformed values.

by Gomez and Gomez (1984) for Randomized Block Design with 3 replicates (3 branches per replication). The statistical analysis was carried out for observed characters by using MS-Excel and OPSTAT software.

RESULTS AND DISCUSSION

Pollen viability (Acetocarmine solution 2% test): Pollen viability is an important aspect of pollen biology and has a direct impact on the adequate fruit setting. The pollen viability varied significantly for the pomegranate germplasm under study. The viability percentage of pollen with acetocarmine solution (2%) was highest (94.29%) in Bhagwa followed by Chawla (91.21%) and the lowest (44.23%) was in Mridula (Table 2). Such variations in pollen viability might be due the effect of germplasm, temperature and their interaction. Pollen quality was also influenced by high temperature, but the effect of higher temperature on pollen viability is cultivar specific (Rahman *et al.* 2013). Similar variations in pollen viability percentage were reported by Melgarejo *et al.* (2000). They reported pollen viability (%) varied from 60.66% in ME5 to 77.05% in ME16 cultivar. Significant differences among five pomegranate cultivars were also reported by Gadge *et al.* (2011) for pollen viability FDA test. The viable pollen ratio in FDA test was higher in cv. Glavas (52.15%) and the lowest in cv. Konjski Zub (36.73%), respectively. Babu *et al.* (2011) observed pollen viability of pomegranate cultivar Ganesh, which ranged from 84.0–95.0% with a mean of 93.2%. Babu *et al.* (2011b) also observed that pollen viability of Bhagwa was found to be about 91.0% with a range of 85.0– 94.0%. Sangma and Singh (2017) recorded pollen viability ranged from 83.34–97.81% in Ganesh, G-137, Mridula, Kandhari Kabuli, Bhagwa and wild pomegranate germplasm accessions. Recently pollen viability was recorded, and observed maximum (97.86%) in G-137 and lowest (85.27%) in Dholka by Kumar and Kaur (2019). The results obtained in our case are not comparable for some cultivars, because there have been changes in the culture medium, incubation time and temperatures. However, the results are very close to those mentioned, varying between (94.29%) for Bhagwa to (44.23%) for Mridula.

Pollen morphology (size and shape): In pomegranate cultivars under study, the length of fresh pollen grains ranged from 13.36 µm in Kandhari Kabuli to 25.75 µm in Chawla (Table 3). The width of fresh pollen grains varied from 9.70 µm in G-137 to 19.79 µm in Mridula. Pollen grains of 8 varieties of pomegranate were circular in shape in microscopic view (Fig 1). Similar type of variations were observed by Varasteh and Arzani (2009). They reported the pollen sizes ranging from 25.86–22.63 µm and 14.47–12.69 µm. Pollen shape for all studied cultivar was prolate (elliptical). Pollen morphology studies on 20 pomegranate cultivars from Shandong province of China indicated that the pollen was prolate spheroid in equatorial view (Yin *et al.* 2011). Yang *et al.* (2015) also studied pollen morphology of pomegranates from different eco-graphical populations in China. They recorded pollen size ranges from 30.51–23.33

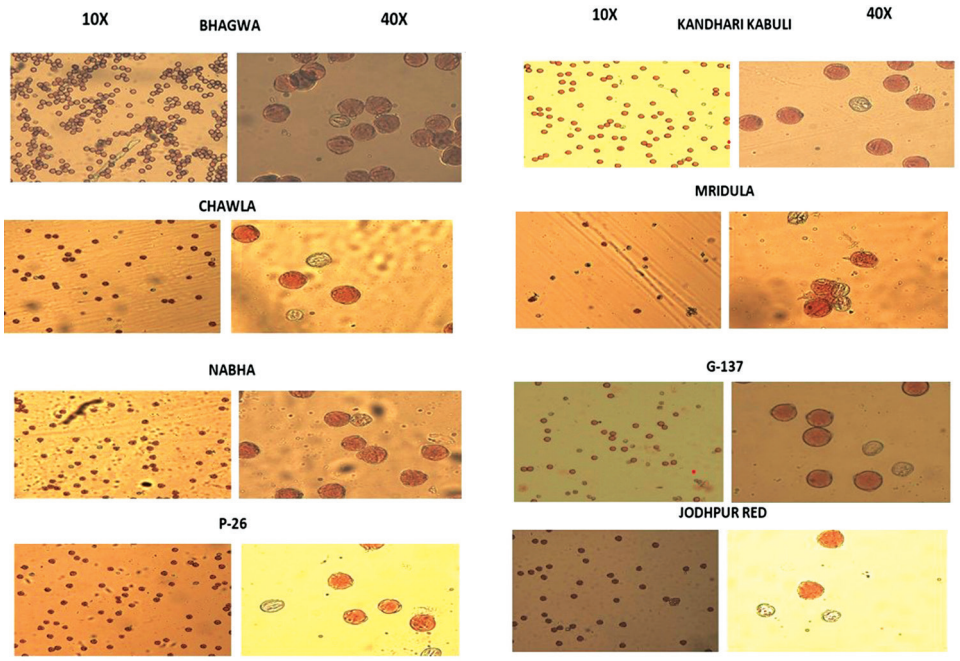

Fig 1 Pollen morphology of different pomegranate cultivars.

µm and 13.07–18.5 µm and pollen shape for all cultivars varied from round, 3 lobed to triangular. Engin and Gobrayak (2019) conducted a study to understand the morphology of pomegranate pollen cv. 'Caner II'. Pollen grains were spherical in shape and approximately 22 µm in size with smooth exine surface. Polar length was from 28.5–26.8 µm whereas pollen grains width was between 15.9–17.1 µm. Our results were in conformity with these reports.

Hybridization studies: Hybridization is a conventional approach of combining desirable traits present in the germplasm. The hybrid progeny is selected in the F_1 or in a later generation after screening for the desirable traits. It can result in a new variety or serve as a breeding line in future breeding programs (Jalikop 2010). In pomegranate, hybridization is relatively easy because of bigger flowers, which makes pollen availability convenient. Additionally, it has better seed germination and most varieties are easily crossable (Jayesh and Kumar 2004).

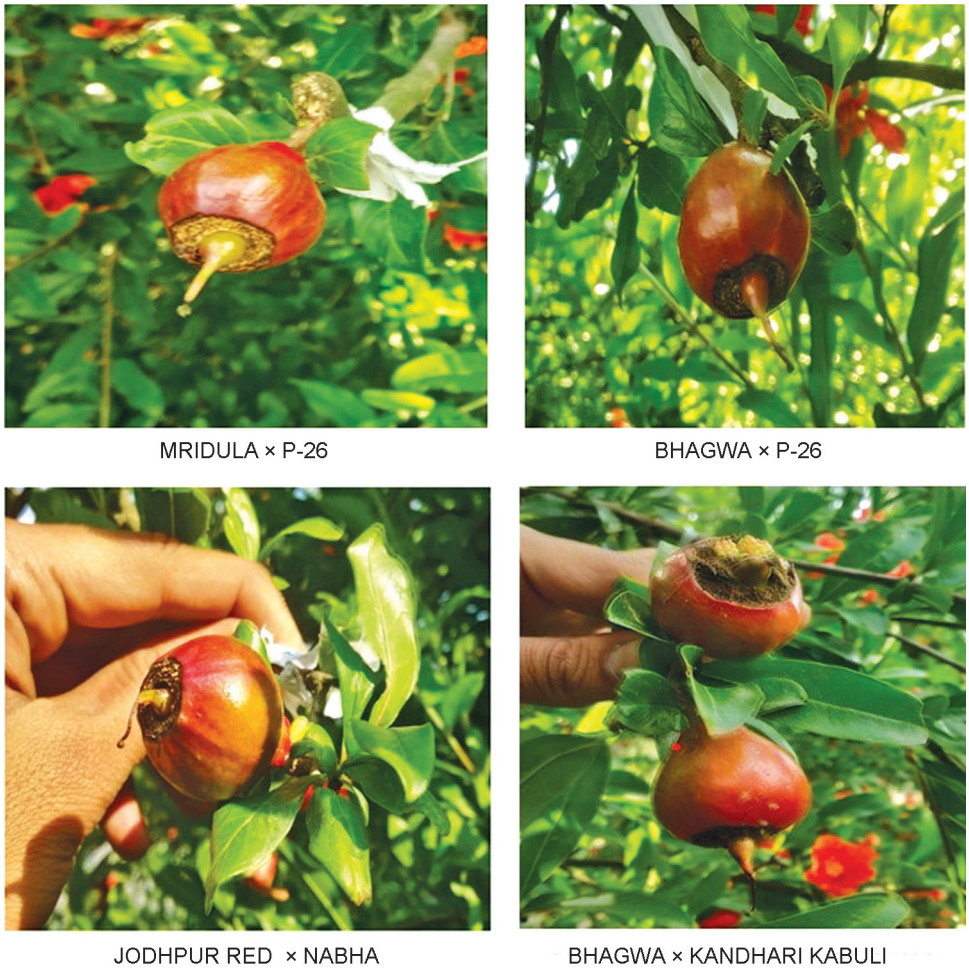

Fig 2 Hybridization fruit set in various cross combinations.

Fruit set percentage is directly dependent on the abundance of flowers and their existence in the plant. Flowering is directly controlled by environment and genetic makeup of the germplasm (Babu *et al.* 2011). In present investigation, the highest fruit set per cent in 16 crosses was observed in cross Mridula × P-26 (77.80%) which was statistically at par with cross Bhagwa × P-26 (66.70%) followed by G-137 × Chawla (36.10%), Jodhpur Red × Nabha (33.33%), Mridula × Nabha (33.33%), G-137 × P-26 (27.77%), Jodhpur Red × Chawla (27.77%), Jodhpur Red × P-26 (22.20%), G-137 × Nabha (22.20%), Bhagwa × Kandhari Kabuli (22.20%), Mridula × Chawla (22.20%), Mridula × Kandhari Kabuli (22.20), G-137 × Kandhari Kabuli (19.43%) and Jodhpur Red × Kandhari Kabuli (11.10%) (Table 3, Fig 2). No fruit set was observed in Bhagwa × Nabha and Bhagwa × Chawla which might be due to the incompatibility in these cross combinations.

In the present investigation, higher fruit sets were obtained when Mridula was used as a female parent as compared to other cultivars. It could be due to variation of genetic makeup and differential response of the germplasm

Table 3 Hybridization fruit set (%) in various cross combination

Cross	Fruit set (%)
Mridula × Kandhari Kabuli	22.20 (23.49)
Mridula × P-26	77.80 (66.49)
Mridula × Nabha	33.33 (29.99)
Mridula × Chawla	22.20 (23.49)
Bhagwa × Kandhari Kabuli	22.20 (23.49)
Bhagwa × P-26	66.70 (54.73)
G-137 × Kandhari Kabuli	19.43 (21.74)
G-137 × P-26	27.77 (26.74)
G-137 × Nabha	22.20 (23.49)
G-137 × Chawla	36.10 (36.73)
Jodhpur Red × Kandhari Kabuli	11.10 (11.74)
Jodhpur Red × P-26	22.20 (23.49)
Jodhpur Red × Nabha	33.33 (29.99)
Jodhpur Red × Chawla	27.77 (26.74)
CD (P=0.05)	31.13

* Figures in the parenthesis are angular transformed values.

to climatic conditions of the locality. Babu *et al.* (2011) studied the crossing pattern in pomegranate where the fruit set was documented 48% in Ganesh with Bhagwa. Babu *et al.* (2011b) also studied the crossing pattern in pomegranate where the fruit set was recorded 35.00% in Bhagwa with Ganesh. Kumar (2012) attempted crosses between soft seeded and hard seeded pomegranate cultivars and found the highest (80.95%) fruit set in Dholka × Kandhari Hansi and minimum fruit set in Dholka × Bush Large (37.60%). All the varieties in which hybridization was done were found to be cross compatible. Bartual *et al.* (2012) conducted hybridization trials to achieve darker skin and early ripening by crossing 'Mollar de Elche' with other foreign varieties. Inter-specific hybrids of 'Mollar' and 'Wonderful' were developed successfully with more fruit set than others. Sangma *et al.* (2017) reported the highest (79.09%) fruit set in Bhagwa × NT-1 and minimum fruit set was recorded in MH-1 × G-137 (57.76%) in a hybridization study between cultivated and wild pomegranate. This significant variation was observed due to adverse climatic conditions resulting in fruit cracking and attack of bacterial blight disease during the hybridization program. In our case, the differences in fruit set percentage could be due to varied degree of compatibility between different genotypes. Behaviour of pollen with respect to viability and germination percentage could also be due to differences in growth rate of pollen tube and might also be due to differences in temperature during bloom period. Moreover, the genetic factors determine the extent of the fruit set and the environmental factors also exercise a strong influence.

In the present investigation, pollen studies in different pomegranate cultivars were adequate with respect to pollen morphology and viability. It can be concluded that the pollen studies could help plant breeders in breeding programs. The pollen viability was found best in cultivars Bhagwa and Chawla which highlights their potential as pollinizers for pollination of commercially growing cultivars. The results indicate that varieties Chawla, Kandhari Kabuli, P-26 and Nabha are the best performing varieties and can be utilized as male parents in the cross combinations in various breeding programs. These studies have demonstrated the suitability of best performing varieties in hybridization and compatibility behavior of cultivars under study. Apprehending pollen production level, viability and fruit set by cross pollination in pomegranate might aid farmers in deciding commercially viable cultivars with higher production value. We believe this study may offer valuable information regarding these subjects.

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