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In vitro Pollen germination of Hermaphrodite Flowers of Wild Pomegranate (*Punica granatum* L.) in Western Himalayas

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ABSTRACT: The study on pollen germination is important for monitoring pollen-stigma interaction, crop improvement, breeding, incompatibility, and fertility studies. The genotypes of wild pomegranate were selected at two locations *viz.*, Tatool (S_1), Solan district and Narag (S_2), Sirmour district of Himachal Pradesh. This species consists of three types of flowers i.e., male, Intermediate and Hermaphrodite. The presented study was carried on pollens collected from hermaphrodite flowers of selected genotypes of wild pomegranate. In vitro pollen germination of hermaphrodite flowers was observed in seven different concentrations of sucrose and boric acid solutions i.e., 5 percent sucrose, 10 percent sucrose, 10 percent sucrose solution + 10 ppm boric acid, 12.5 percent sucrose solution, 12.5 percent sucrose solution + 10 ppm boric acid, 15 percent sucrose and 20 percent sucrose to find the best sucrose concentrations for germination. The germinated pollen was counted for 48 hours until there was no further germination recorded. Two-way ANOVA and factorial design were performed for statistical analysis. The average germination percentage was obtained largest i.e., 83.57 percent in a mix solution of 12.5% of sucrose + 10 ppm of boric acid and the least 15.23 percent and 19.5 percent in a solution of 20 % and 5% sucrose solution respectively. Statistically significant difference was obtained for pollen germination among the selected genotypes and their interactions.

Keywords: pollen germination, boric acid, sucrose, hermaphrodite flower, wild pomegranate.

INTRODUCTION

The wild Pomegranate (*Punica granatum* L.) belonging to the order Myrtales and Lythraceae family is one of the ancient edible fruits, native to Iran and grows naturally in the foothills of Western Himalayas (Rana *et al*., 2007; Singh *et al*., 2015). Wild pomegranate (*Punica granatum* L.), Vern. Daru can grow in a variety of agro-climatic conditions ranging from tropical to subtropical countries (Levin, 2006 and Jalikop, 2007). There are two subspecies of granatum; *Punica granatum* ssp. *chlorocarpa* is native to the Transcaucasian region and *Punica granatum* ssp. *porphyrocarpa* is native to Central Asia. (Sharma *et al*., 2009), has also spread to the Himalayas in Northern India (Mars, 2000). The Western Himalayas show the availability of a very diverse collection of wild pomegranates (Rana *et al*. 2007). It is considered as a proto type of cultivated one and resembles cultivated pomegranate for various morphological characters. In India, wild pomegranate grows only in three states: Jammu and Kashmir, Himachal Pradesh and Uttarakhand (Narzary *et al*., 2009; Mahajan *et al.* 2018). There is a growing demand of wild pomegranate for good quality fruits both for fresh use and for making anardana, which are the dried arils of wild pomegranate with a distinct sour flavour and are mostly consumed in culinary preparations. (Murtaza and Ahmad 2017). Anardana is also used as a source of livelihood by the locals. Presence of secondary metabolites in different plant parts of wild pomegranate and high antioxidant activity is responsible for its health benefits and medicinal properties (Kaur *et al.*, 2018). Himalayan variety has resistance to the deadly bacterial blight but these are not high yielding whereas cultivated variety are high yielding but susceptible to bacterial blight, it resulted in yield losses up to 60-80% (Sharma *et al*., 2015). Indian Institute of Horticulture Research, revealed that not only the wild types but the hybrids of both cultivated and wild types are found to be resistant against the bacterial disease (Jalikop, 2005). In addition, the commercial marketing of pomegranate is still limited by physiological disorders such as rind cracks, cold damage, skin burns and excessive weight loss (Caleb *et al*., 2012). The wild pomegranate has a thick fruit skin which makes it less susceptible to butterfly attacks and cracking, So the breeding for these traits becomes more important (Jalikop *et al*., 2010). It has three types of flowers viz; pins, thrum and homostyle (Singh *et al*., 2006), which differ in their

pollen viability and germination. Pollen, the male gametophyte, is an evolutionary development in higher plants that ensures successful genetic exchange, establishment, and survival of the species (Ashman *et al*., 2004; Pacini and Dolferus, 2016). Because of their crucial role in successful seed development Rosbakh *et al.*, 2018), pollen germination (PG), and pollen tube growth (PTG) have been in the focus of many studies ranging from research on physiological and biochemical aspects of these processes (Williams and Reese, 2019) to large-scale screenings of pollen abiotic stress-tolerance (Rosbakh *et al.*, 2018). In basic and applied research, pollen functioning has been studied with the help of two approaches, *in vivo* and *in vitro*. *In vivo* studies are carried out directly at the stigmatic surface in the natural state, while *in vitro* approaches rely on a culture medium that simulates conditions of the style-stigma (Rodriguez-Enriquez *et al*., 2013). The advantage of the *in vivo* methods is that they consider all natural conditions pollen grains experience on stigma (Albert *et al.*, 2018). The *in vitro* approach is generally preferred because it provides results comparable to *in vivo* studies (Sulusoglu and Cavusoglu, 2014; Jayaprakash, 2018; Luo *et al*., 2020). In some cases, other sugars and sugar derivatives such as lactose, maltose, raffinose, and fructose among others, are also used (Shaoling *et al*., 2005; Hirsche *et al*., 2017; Lagera *et al.*, 2017; Impe *et al.*, 2019). Therefore, detailed studies on the pollen germination are essential for monitoring pollen vigor during storage, genetics, and pollen-stigma interaction studies, crop improvement and breeding, and incompatibility and fertility studies. It is one of the important factors for fertilization success and have a significant role in pollination for adequate fruit yield (Sutyemez, 2011; Nikolic *et al*., 2012).

MATERIALS AND METHODS:

The study was carried out in the lab of Department of Tree Improvement and Genetic Resources, College of Forestry, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh. Two locations were selected viz; Tatool (S_1) in district Solan and Narag (S_2) in district Sirmour of Himachal Pradesh. At each location, five morphologically best trees/genotypes were randomly selected for pollen collection. Pollen was collected of hermaphrodite flowers only. In – vitro pollen germination was observed in five different concentrations of sucrose and boric acid solutions for 48 hours later until there was no further germination. Two – way ANOVA and factorial design were used for the statistical analysis.

Pollen sample collection: At the time of anthesis, fifty hermaphrodite flowers were identified and tagged on each selected tree. During month of March-April, the tagged hermaphrodite flowers ready for pollen dehiscence were enclosed in separate butter paper bags overnight. These freshly released pollen was collected in the next morning to study pollen germination. The mature inflorescences in the pre-anthesis phase were also collected and kept in partial shade under lab conditions. Pollens of these mature inflorescence collected on sheet of paper on dehiscence. All pollen samples of different genotypes were collected separately to avoid contamination from other sources. The pollen grains thus collected were placed in glass vials and stored in the refrigerator at minimum temperature.

In-vitro pollen germination: The freshly dehisced pollen grains were used for germination experiments. In this test, the pollens of hermaphroditic flowers were used separately of different trees/genotypes and tested with different concentrations of sucrose. The different solutions used were:

(a) 5 per cent sucrose solution
(b) 10 per cent sucrose solution
(c) 10 per cent sucrose solution + 10 ppm boric acid
(d) 12.5 per cent sucrose solution
(e) 12.5 per cent sucrose solution + 10 ppm boric acid
(f) 15 per cent sucrose solution
(g) 20 per cent sucrose solution

The pollen grains were placed on cavity slides containing a hanging drop of sucrose solution and the growth of the pollen tube in each culture was evaluated under a microscope. It has been assumed that pollen grains have a pollen tube that is at least four times the size of germinated pollen. The pollen germination in different concentrations was observed for 48 hours later until there was no further germination.

Analysis of variance (ANOVA). For working out the analysis of variance, the data were analysed by using the following model as suggested by Panse and Sukhatma (1967).

$$\mathbf{Y_{ij} = \mu + gi + bj + eij}$$

i = 1,2,.....g
j = 1,2,.....r

where,

Y_{ij} = phenotypic observation of i^{th} entry and j^{th} replication
μ = general mean of the population
g_i = effect of i^{th} genotype
b_j = effect of j^{th} replication, and
e_{ij} = error component

Table 1.

Source of variation	d.f	Sum of Square	Mean of squares	Expectation
Replication	(r-1)	$1/g \Sigma jy\, j^2 - C.F.$	M_r	—
Genotype	(g-1)	$1/r\ \Sigma iy\, j^2 - C.F.$	M_g	$\sigma^2 e + r\sigma^2 g$
Error	(r-1) (g-1)	By substraction	Me	$\sigma^2 e$
Total	**(rg-1)**	—	—	—

Where,
r = No. of replications
g = No. of genotypes
M_r = Mean sum of squares due to replications
M_t = Mean sum of squares due to genotypes
M_e = Mean sum of squares due to error.

Critical difference (CD): The critical difference (CD) was calculated as under:

CD = $SE_d \times t_{0.05}$ error degree of freedom

Where;

SE_d = Standard error of difference calculated as:

$SE_d = \sqrt{2M_e / r}$

$t_{0.05}$ error degree of freedom = t value at 5 per cent level of significance.

RESULTS AND DISCUSSION

The germination of pollen collected from hermaphroditic flowers was tested in seven different sucrose solutions. The results of pollen germination were observed significantly differ for tree, concentration, location × tree, location × concentration, tree × concentration, and location × tree × concentration, locations, trees, flower types and their interactions.

Table 2: Pollen germination (%) in pomegranate flowers.

Treatment	(S_1) Tatool (Tree No.)						S_2 Narag (Tree No.)					
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	Mean	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	Mean
5% sucrose	34.41 (35.9)	19.5 (26.19)	21.63 (27.7)	25.27 (30.16)	35.17 (36.36)	27.19 (31.26)	22.4 (28.24)	21.5 (27.61)	25.27 (30.16)	35.5 (36.56)	35.17 (36.36)	27.97 31.79)
10% sucrose	45.2 (42.23)	21.33 (27.49)	22.7 (28.44)	30.57 (33.55)	46.65 (43.06)	33.29 (34.96)	35 (36.26)	33.57 (35.39)	36.73 (37.29)	49.4 (44.64)	47.33 (43.45)	40.41 (39.41)
10%surose+10 ppm boric acid	55.9 (48.37)	35.8 (36.74)	37.66 (37.84)	40.47 (39.49)	58.4 (49.82)	45.65 (42.45)	52.6 (46.47)	51.13 (45.63)	53.1 (46.76)	56.37 (48.64)	55.47 (48.12)	53.73 (47.12)
12.5% sucrose	58.37 (49.8)	43.1 (41.02)	48.5 (44.12)	52.47 (46.39)	60.47 (51.02)	52.58 (46.47)	55.4 (48.08)	54.3 (47.45)	56.23 (48.56)	60.17 (50.85)	58.47 (49.86)	56.91 (48.96)
12.5% sucrose + 10ppm boric acid	82.23 (65.04)	69.23 (56.29)	70.43 (57.04)	72.43 (58.31)	83.2 (65.78)	75.51 (60.49)	73 (58.67)	69.07 (56.18)	74.83 (59.87)	83.57 (66.06)	83.2 (65.78)	76.73 (61.31)
15% sucrose	75.2 (60.11)	60.47 (51.02)	62.3 (52.1)	64.5 (53.41)	77.3 (61.52)	67.95 (55.63)	68.23 (55.67)	65.17 (53.81)	69.53 (56.48)	78.17 (62.12)	79.13 (62.79)	72.05 (58.17)
20% sucrose	30.17 (33.3)	20.4 (26.84)	15.23 (22.97)	27.6(31.68)	29.2 (32.69)	24.52 (29.5)	32.67 (34.85)	26.33 (30.86)	25.1 (30.06)	28.27 (32.11	23.2 (28.78)	27.11 (31.33)
Mean	54.5 (47.82)	38.55 (37.94)	39.78 (38.6)	44.76 (41.86)	55.77 (48.61)	46.67 (42.97)	48.47 (44.03)	45.87 (42.42)	48.69 (44.17)	55.92 (48.71)	54.57 (47.88)	50.7 (45.44)

* Figures in parenthesis are angular transformed values.

CD Site	Tree	Concentration	Site×Tree	Site × oncentration	Tree × concentration	Site × Tree × concentration
0.13	0.21	0.24	0.29	0.34	0.54	0.77

Among the different concentrations, the maximum average germination percentage (76.73%) was observed with 12.5% sucrose + 10 ppm boric acid and the minimum 24.52% with a 20% sucrose solution. In the Site × Tree interaction, the maximum percentage observed was 55.92% in S_2T_4 and 55.77% in S_1T_5 and the minimum was observed in S_1T_2 (38.55%). In the site × concentration interaction, the maximum mean percentage observed was 76.73% at Narag with a solution of 12.5% sucrose + 10 ppm boric acid and the minimum value was 27.11% at Narag with a solution of 20% sucrose. Regarding the location × tree × concentration, the maximum percentage was 83.57% in S_2T_4 with 12.5% sucrose + 10 ppm boron and the minimum percentage of pollen germination was observed in S_1T_2 (19.5%) with 5% sucrose (Fig. 1). The significant results for pollen germination led to the conclusion that more than 50 percent of the germination percentage is observed in pollens of hermaphrodite flowers of this species. Pollen viability and germination studies can be used in crop breeding which create cultivars with high yield, large fruits, resistance to insect pest and diseases and low cost of production (Sharma *et al*., 2021). Dinesh *et al*., (2017) examined the maximum germination of pollen in a 10 percent sucrose solution after 72 hours with a germination of 42.34% and a minimum (2.17%) in a sucrose solution at 20% in varieties of pomegranate and wild germ plasma adhesions. Mishra *et al*., (2020) reported the maximum germination of wild pomegranate pollen for Mandi district (62.20%) and the minimum for Sirmour (56.55%) in the medium with 15% sucrose and 10 ppm boric acid.

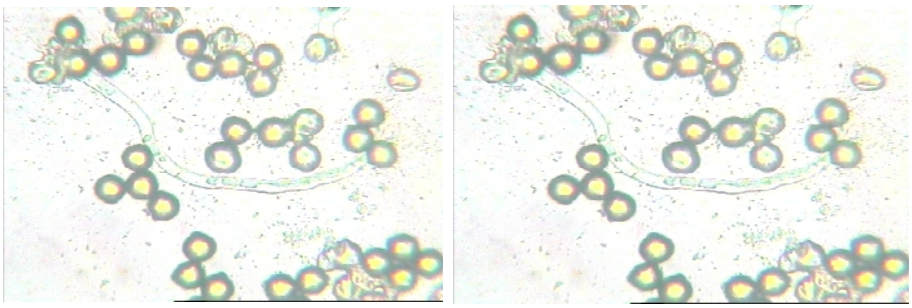

Fig. 1. Pollen germination in 12.5 % Sucrose + 10 ppm Boric acid solution.

CONCLUSION

Reproduction rate, fruit production of this species depends upon the proportion of fertile flowers (hermaphrodites), percentage of pollen germination. As this species comprised of and romonoecious types of flowers i.e., male and hermaphrodite types of flowers. Hermaphrodite flowers are more fertile than male flowers. Therefore, the use of pollens of hermaphroditic flowers for reproduction, breeding purposes can gives a higher success. Since very few studies have been conducted on wild pomegranate, it can be used by the breeder as a guide for the development of highly productive disease resistant strains of wild pomegranate. Also, development of cross hybrids of cultivated and wild types. Here we identified more than 50 percent rate of germination of pollen collected from hermaphrodite flowers. In-vitro pollen germination is also a reliable method to test the pollen viability. It also addresses many basic questions in sexual reproduction and particularly useful in wide hybridization.

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Conflict of Interest. Authors have declared that no competing interest exist.

REFERENCES

Albert, B., Ressayre, A., Dillmann, C., Carlson, A. L., Swanson, R. J., Gouyon, P. H., & Dobritsa, A. A. (2018). Effect of aperture number on pollen germination, survival and reproductive success in Arabidopsis thaliana. *Annals of Botany, 121*(4): 733-740.

Ashman, T. L., Knight, T. M., Steets, J. A., Amarasekare, P., Burd, M., & Campbell, D. R. (2004). Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology, 85*: 2408–2421.

Caleb, O. J., Mahajan, P. V., Opara, U. L., & Witthuhn, C. R. (2012). Modeling the effect of time and temperature on respiration rate of pomegranate arils (cv. 'Acco' and 'Herskawitz'). *Journal of Food Science, 77*: 80–87.

Nikolic, D., Rakonjac, S., & Fotiric-Aksic, M. M. (2012). The effect of pollinizer on the fruit set of plum cultivars Cacanska Najbolja. *Journal of Agricultural Science, 57*: 9–18.

Dinesh, S., Donald, S., & Kumar, K. (2017). Hybridization studies on pomegranate (*Punica granatum* L) cultivars and wild germplasm accessions. *International Journal of Farm Sciences, 7*: 119-126.

Hirsche, J., García Fernández, J. M., Stabentheiner, E., Großkinsky, D. K., & Roitsch, T. (2017). Differential effects of carbohydrates on *Arabidopsis* pollen germination. *Plant Cell Physiol., 58*: 691–701.

Impe, D., Reitz, J., Köpnick, C., Rolletschek, H., Börner, A., & Senula, A. (2019). Assessment of pollen viability for wheat. *Front. Plant Sci., 10*: 1588.

Jalikop, S. H., Venugopalan, R., & Kumar, R. (2010). Association of fruit triats and aril browning in pomegranate (*Punica granatum* L.) *Euphytica, 174*: 137-141.

Jalikop, S. H. (2007). Linked dominant alleles of inter – locus interaction results in a major shift in pomegranate fruit acidity of Ganesh and Kabul yellow, *Euphytica, 158*: 201-207.

Jalikop, S. H., Rawal, R. D., & Kumar, R. (2005). Exploitation of sub-temperate pomegranate daru in breeding tropical varieties. *Acta Horticulture, 696*: 107-112.

Jayaprakash, P. (2018). "Pollen germination *in vitro*," in *Pollination in plants*, ed. P. W. Mokwala (Chennai: InTech).

Kaur, R., Aslam, L., Kapoor, N., & Mahajan, R. (2018). Phytochemical analysis and antioxidant activity of wild pomegranate collected from Patnitop, Jammu & Kashmir. *Biosci., Biotechnol. Res. Asia1., 5*: 335–341.

Lagera, A. J., Balinado, L.O., Baldomero, J. R., Rotairo, H. F. I., Tero, N. L., & Maghinay, M. S. (2017). Varying sugars and sugar concentrations influence *in vitro* pollen germination and pollen tube growth of *Cassia alata* L. *J. Young Investig., 1*: 42–45.

Levin, G. M. (2006). Pomegranate 1st edition, third millennium publishing, East Libra Drive Tempe, AZ, 13-120.

Luo, S., Zhang, K., Zhong, W. P., Chen, P., Fan, X. M., & Yuan, D. Y. (2020). Optimization of *in vitro* pollen germination and pollen viability tests for *Castanea mollissima* and *Castanea henryi*. *Scientia Horticulturae, 271*: 109481.

Sutyemez, M. (2011). Pollen quality, quantity and fruit set of some self compatible and self-incompatible cherry cultivars with artificial pollination. *African Journal of Biotechnology, 10:* 3380–3386.

Mahajan, R., Javed, A., & Kapoor, N. (2018). Characterization of genetic diversity of wild pomegranate collected from Himachal Pradesh, India. *Ann Plant Sci., 7*: 2042–2046.

Mars, M. (2000). Pomegranate plant material genetic resources and breeding, a review. *Option Mediterranean Series A., 45*: 105-113.

Mishra, G., Karna, A., Hota, D., & Sharam, G. (2020). In vitro pollen germination potential of wild pomegranate (*Punica granatum* L.) genotypes in western Himalayan region. *International Journal of Chemical Studies, 8*(4): 2385-2388.

Murtaza, M. S., & Ahmad, G. S. (2017). Anardana (dehydrated wild pomegranate arils) as livelihood option for rural communities in Chenab valley of Jammu and Kashmir. *Indian J. Hort., 74*: 306–309.

Narzary, D., Mahar, K. S., Rana. T. S., & Ranade, S.A. (2009). Analysis of genetic diversity among wild pomegranates in Western Himalayas using PCR methods. *Scientia Horticulture, 121*: 237-242

Pacini, E., & Dolferus, R. (2016). "The trials and tribulations of the plant male gametophyte — understanding reproductive stage stress tolerance," in *Abiotic and biotic stress in plants - recent advances and future perspectives*, eds A. K. Shanker and C. Shanker (Chennai: InTech).

Panse, V. G., & Sukhatme, P. V. (1967). Statistical Methods for Agricultural Workers. ICAR, New Delhi, 610 p.

Rana, J. C., Pradeep, K., & Verma, V. D. (2007). Naturally occurring wild relatives of temperate fruits of western Himalayan region of India: An analysis. *Biodiversity Conservation, 16:* 3963-3991.

Rodriguez-Enriquez, M. J., Mehdi, S., Dickinson, H. G., & Grant-Downton, R. T. (2013). A novel method for efficient *in vitro* germination and tube growth of *Arabidopsis thaliana* pollen. *New Phytol., 197*: 668–679.

Rosbakh, S., Pacini, E., Nepi, M., & Poschlod, P. (2018). An unexplored side of regeneration niche: seed quantity and quality are determined by the effect of

temperature on pollen performance. *Front. Plant Sci.*, *9*: 1036.

Shaoling, Z., DiXin, C., Lang, K., & Ling, W. (2005). Effects of medium components and pH on pollen germination and tube growth in pear (*Pyrus pyrifolia*). *Acta Botanica Boreali-Occidentalia Sinica*, *25*: 225–230.

Sharma, K. K., Sharma, J., & Jadhav, V. T. (2015). Book: Recent development in bacterial blight of pomegranate and its management. Pp.119-126.

Sharma, V. K., Goswami, M., Sharma, S. D., & Attri, K. (2021). Pollen viability and Pollen Germination Studies in Low Chill Peach [*Prunus persica* (L.) Batsch] Cultivars in the Subtropics of North-West Himalayas. *Biological Forum - An international Journal (Research Trend), 13*(1): 746-750.

Sharma, G., Sharma, O. C., & Thakur, B. S. (2009). Pomegranate. Systematics of Fruit Crops. NEW India Publishing Agency, New Delhi, pp. 257-262.

Singh, N. V., Abburi, V. L., & Ramajayam, D. (2015). Genetic diversity and association mapping of bacterial blight and other horticulturally important traits with microsatellite markers in pomegranate from India. *Mol. Genet. Genom., 290*: 1393–1402.

Singh, N. P., Dhillon, W. S., Sharma, K. K., & Dhatt, A. S. (2006). Effect of mechanical deblossoming on flowering and fruit set in pomegranate cultivars: Ganesh-I and Kandhari. *Indian Journal of Horticulture*, *63*: 383-385.

Sulusoglu, M., & Cavusoglu, A. (2014). *In vitro* pollen viability and pollen germination in cherry laurel (*Prunus laurocerasus* L.). *Sci. World J.*, 2014:657123. doi: 10.1155/2014/657123**.**

Williams, J. H., & Reese, J. B. (2019). Evolution of development of pollen performance. *Curr. Dev. Biol.*, *131*: 299–336.

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