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ORIGINAL ARTICLE

Comparative Study of Some Important Reproductive Features in Two Populations of *Podophyllum Hexandrum* Royle, an Endangered Plant of Kashmir Himalaya

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ABSTRACT
The Himalayan region is bestowed with high-value medicinal plants, including Podophyllum hexandrum Royle. It belongs to the family Berberidaceae and the order Ranunculales. The present study investigated female gametophyte development, pollen and seed viability, and seed germination in two populations of Podophyllum hexandrum from Yaripathri Kulgam and Hirpura Shopian. The P. hexandrum ovule was anatropous, bitegmic, and psudocrassinucillate, and the outer integument contributed more to micropyle development, exhibiting steps towards advancement in the evolutionary process. Moreover, comparing the pollen and seed viability of two populations using Lugol's iodine solution and 1% TCC solution showed the maximum percentage of pollen and seed viability in populations from low-altitude areas (Yaripathri). In addition, seed germination in both populations using IAA, IBA, and GA3 showed the maximum germination percentage in GA3-treated seeds.
Keywords: *Podophyllum hexandrum, female gametophyte, pollen viability, pollen germination, seed germination and viability*

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INTRODUCTION

Podophyllum hexandrum Royle, commonly known as Indian May Apple (Berberidaceae), is one of the valuable medicinal plants natives to the Himalayan area. It is found in restricted areas in the Himalayan region at elevations between 1300 and 4300 m above sea level [1]. The herbaceous plant, *P. hexandrum,* has a large rhizomatous system that enables it to propagate and endure extremely cold conditions, and it sprouts into young seedlings when favourable climatic conditions appear. In the spring, white or pale pink, 6-petaled flowers are borne at the ends of stout stems; fleshy, oval, red berries follow these. The flower in May-August has six petals and six stamens, which inspired its species name, *hexandrum*, meaning six stamens. Leaves are rounded in outline, 10-25 cm long, deeply cut into 3 ovate, toothed lobes, sometimes further lobed. Fruit is a large scarlet or reddish berry, 2.5-5 cm, with many seeds embedded in pulp. It can be propagated by seed or by dividing the rhizome [2, 3]. It has been utilized in Kashmir's traditional medical system for ages and is locally known as Banwangun because its red fruit (berry) is about the size of a small brinjal. It is renowned for its anticancer properties [4]. *P. hexandrum*'s rhizomes and roots contain antitumor lignans such as podophyllotoxin, 4'-dimethyl podophyllotoxin, and podophyllotoxin 4-o-glucoside [4]. Additionally, it has been shown that aryl tetralin lignan regulates the synthesis of steroidal hormones, thus lowering the risk of breast and prostate cancer [5, 6]. *P. hexandrum* has a relatively small and diminishing population (40–700 plants per site). A portion of the population in certain areas has almost entirely vanished due to human actions and overexploitation [7]. Since seed germination and seedling establishment are comparatively lower, rhizomes are the primary means of multiplication in natural areas. The species requires immediate conservation attention since it is already in danger of disappearing and because the rate at which its subterranean parts are exploited increases

that of natural regeneration [1]. In this respect, research on its reproductive biology and genetic diversity is crucial for effectively designing conservation measures. *P. hexandrum* from the heart of the Himalayas exhibits significant variation in morphological traits, including plant height, leaf attributes, fruit weight, seed weight, and color [8, 9]. `

Two locations (Yaripathri Kulgam and Hirpura Shopian, Jammu and Kashmir) were chosen to investigate the pollen viability, seed viability, seed germination, and morphological and anatomical differences among the wild populations of *P. hexandrum* collected from these two study areas and their relationship with geographical altitude to provide insight to facilitate conservation management of the remaining populations. Appropriate conservation management should be adopted, including in situ conservation and germplasm collection from the remaining populations with the greatest genetic variation.

MATERIAL AND METHODS

Pollen viability

Two naturally occurring populations of *P. hexandrum* were investigated, one from Yaripathri Kulgam, located at an elevation of 1659 m in the thicket of small herbs. The population was dense and had a larger number of individuals. Second from Hirpura Shopian, located at the height of 2678 m in *Pinus* forest. For the study of Pollen viability and embryological assessment, Flowers and flower buds were collected, preserved in Carnoy's solution (ethanol and glacial acetic acid in a 3:1 ratio), and stored at 4^{o}C for further examination.

Starch content assessment

Anthers from flowers were crushed to release pollen grains on a slide to examine the presence of starch in the pollen grains. A drop of iodine-potassium iodine (Lugol's iodine solution) was applied to the pollen. Pollens were quickly detected under a compound microscope. Deeply stained pollens (black in color) were thought to accumulate more starch than less stained pollens. The same test was also used to determine pollen viability.

Embryo viability assessment

The Tetrazolium test was used to measure embryo viability. Before starting the test, mature seeds of two examined populations were imbibed on filter paper moistened with distilled water overnight at 20-25°C. The entire procedure was carried out in a Petri dish. The seeds were then split into equal halves longitudinally and incubated in a 1% solution of 2, 3, and 5-triphenyl tetrazolium chloride (TTC). According to Lakon, all respiring biological tissues are capable of converting a colorless chemical (2, 3, 5-triphenyl tetrazolium chloride or bromide) into a red compound Formazen via H^+ transfer processes catalyzed by the enzyme dehydrogenases. Because formazan is non-diffusible, it stains living tissues red. As a result, the living sections of viable seeds stained red (the darker the color, the greater the respiration rate in the seed). The embryo viability was assessed using stain intensity:

1) The seed is viable if the embryo and the endosperm are stained.
2) If the seeds are not stained, or if any portion of the seed (embryo/endosperm) is not stained, the seed is considered non-viable.

Estimation of germination rate

Observations were recorded, and photographs were taken under Stereo Zoom Carl Zeiss Microscope. To estimate the germination rate, mature seeds of *P. hexandrum* were collected from the study areas (Yaripathri Kulgam and Hirpura Shopian). The collected seeds were surface sterilized by treating seeds first with fungicide (0.01% Bavistin) for 1 hour. These seeds were then washed with running tap water for 5 minutes. 2-3 drops of Labollene (Detergent) were added to the beaker containing these seeds, and the open side was covered with a muslin cloth so that running tap water would pass through it for 10 minutes.

In addition to this, 1-2 drops of Tween 20 were added through muslin cloth and washed with running water for 20 minutes. Seeds were dried and treated with 1% sodium hypochlorite (NaOCl) for 10 minutes. Seeds were washed and autoclaved with double distilled water. Seeds were washed with 70% ethanol for 30-45 seconds and put on autoclaved Whatman's No.1 filter paper for drying. The physical scarification was done by soaking *P. hexandrum* seeds in distilled water for 24 hours at room temperature (25°C) and then treated with hot distilled water (80°C) for 2 minutes. Also, the seeds were dipped in concentrated H_2SO_4 for 10 seconds. The seeds were divided into 4 groups, each containing 50 seeds. One group of seeds were controlled, and the other 3 groups were treated with different plant growth regulators. According to seed testing association (ISTA) protocol 1985, seeds were treated with different plant growth regulators for 10 minutes, IAA@100ppm, IBA@100ppm and GA3@100ppm concentration. After giving respective treatments, seeds were spread in Petri plates lined with moistened filter paper, and the time was noted. The data was collected in the interval of 50 days.

RESULTS

The features uncovered during the anatomy and female gametophyte development of two native populations of *P. hexandrum* were the same, but there was a difference in seed viability and germination.

Ovule and development of female gametophyte

Both the populations of *P. hexandrum* bore cup-shaped, hermaphrodite, actinomorphic and gamosepalous flowers. These flowers had superior, unilocular, monocarpellary ovaries with 50-160 ovules with marginal placentation. Mature ovules were anatropous with two integuments (bitegmic) and pseudo-crassinucillate (Fig 1). Ovules began as homogenous tissue masses on the central placenta and appeared to develop similarly to orthotropous ovules. Embryo sac development found was polygonum monosporic type, involving a single archesporial cell differentiating into a megaspore mother cell (MMC) that followed the meiotic path to create a linear tetrad of megaspores (Fig 2). The chalazal megaspore (the other three degenerated) divided three times to form a 7-celled, 8-nucleated embryo sac (Fig 3). A little cavity was above the egg apparatus towards the developed embryo sac micropyle. Egg cells had a nucleus (pushed towards the chalazal end) and a big vacuole. The embryo sac's micropylar end had larger synergids. Polar nuclei merge into secondary nuclei. The egg apparatus is near the secondary nucleus. Nucellar cells degenerate as the embryo sac grows.

Fig 1: Ovules inside ovary

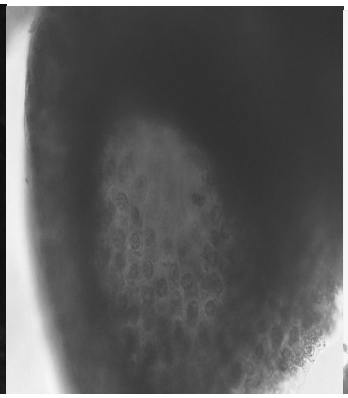

Fig 2: MMC's within ovule

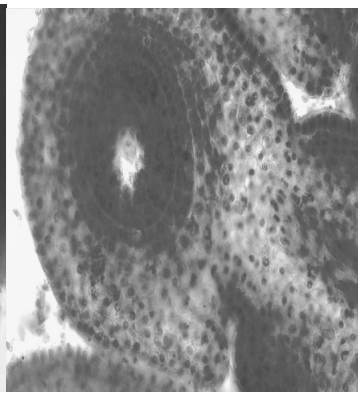

Fig 3: Embryo sac

Pollen viability

The pollen viability of the two populations was tested using Lugol's solution. *P. hexandrum* releases pollen grains in four-celled tetrad form.

In both populations, most pollen tetrads were stained deeply and uniformly, confirming that the whole tetrad was viable. Fully non-viable tetrad was also observed in the specimen collected from Hirpura, Shopian.

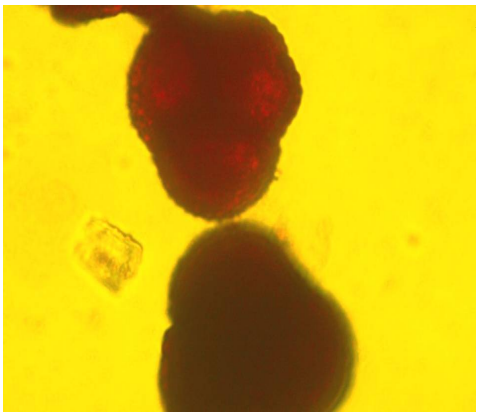

Fig 4: one lightly stained (non-viable) and one darkly stained (viable) tetrad after Lugol's Iodine solution.

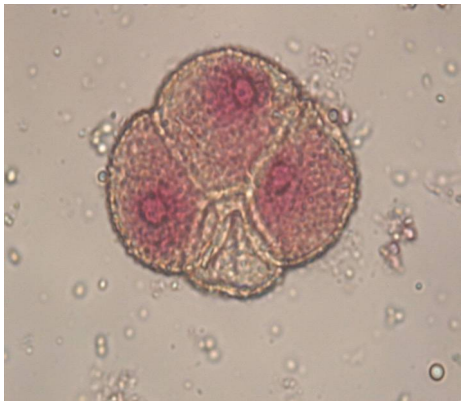

Fig 5: Tetrad without Lugol solution treatment

Seed viability

The viability of seeds was analyzed by treating seeds with 1% TCC solution; as a result, seeds were divided into three classes based on the staining intensity of treated seeds. Class I**:** Seeds completely stained pink were considered viable (Fig 6). Class II: Seeds stained partially were considered non-viable

(Fig 8). Class III: Unstained Seeds were regarded as non-viable (Fig 9). Further, embryos that stained pink with 1% TCC solution were viable, and those that remained unstained were non-viable (Fig 7 and 10). The maximum seed viability percentage was found in seeds of the Yaripathri Kulgam population. (Table 1).

Table1. Estimation of Seed viability as per AOSA rules for TZ testing

Location	Total number of examined seeds	Number of viable seeds	Viability percentage
Yaripathri Kulgam	100	89	89%
Hirpura Shopian	100	78	72%

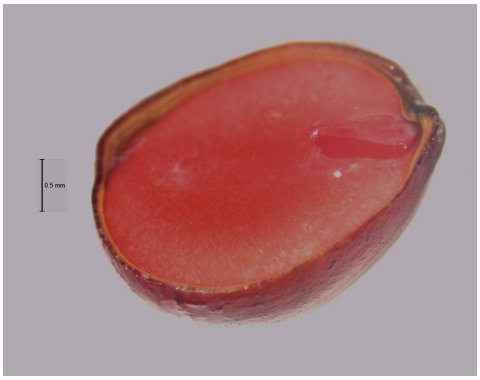

Fig 6: Class I Mature viable seed after TZ treatment.

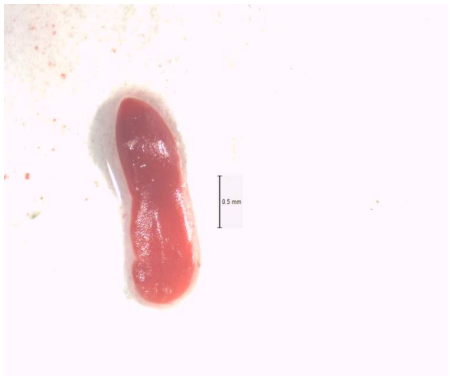

Fig 7: Viable embryo after TZ

Fig 8: Class II Partially stained non-viable

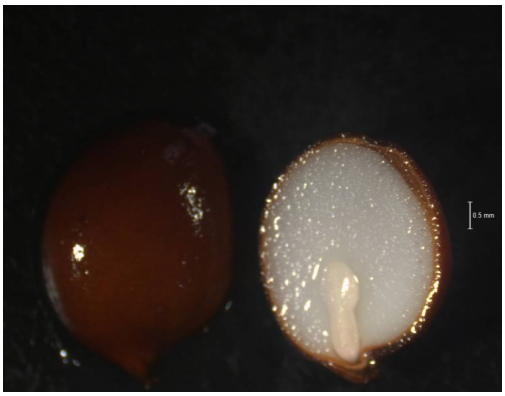

Fig 9: Non-viable seed after TZ testing.

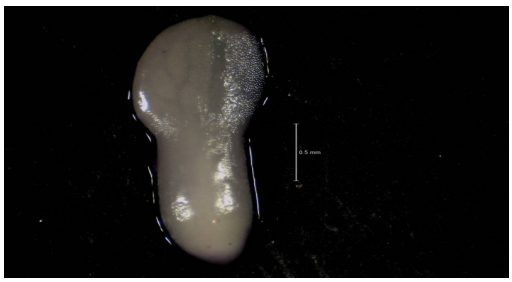

Fig 10: Non-viable embryo after TZ testing

Seed germination

The seed germination times varied in both Yaripathri Kulgam and Hirpura, Shopian populations. The time taken to germinate on moistened filter paper was 36-58 days. The chemically scarified seeds and treated with different plant growth regulators (PGR) were taken to check dormancy. Seeds treated with IAA, IBA, and GA3 exhibited a higher germination percentage than the control. The germination percentages of seeds from the Yaripathri Kulgam area were higher than the seeds from Hirpura, Shopian

area. The seed germination under control conditions was found at 48% for seeds from and 39% for seeds from. IAA-treated seeds showed 53% and 46% germination, and IBA-treated seeds showed 58% and 51% germination for seeds from Yaripathri Kulgam and Hirpura Shopian, respectively. GA3 treated seeds showed the highest germination of 69% and 62% for seeds from Yaripathri Kulgam and Hirpura Shopian, respectively (Table 2).

Table2: Seed germination percentage after PGR treatment.

Study area	Control	IAA	IBA	GA3	Days taken to germinate
Yaripathri Kulgam	48%	53%	58%	69%	40±2
Hirpura Shopian	39%	46%	50%	62%	45±5

DISCUSSION

P. hexandrum is a member of the Berberidaceae family, which is closely related to Ranunculaceae, Manispermaceae, and Lardizabalaceae (all Ranunculales). Primitive Ranunculales are fascinating. Bitegmic, anatropous, and crassinucellate ovules are primitive compared to unitegmic, ategmic, and tenuinucellur ovules, widespread in angiosperms (Bouman 1984). In numerous families of Ranunculales, crassinucellate embryo sac development is documented [10-12]. *P. hexandrum* ovule development is Psudocrassinucillate [13].

The *Podophyllum hexandrum* plant is rare yet highly prized for its medicinal properties. Medicinal ingredients, including podophyllotoxin and lignans, are typically extracted from the rhizome. As a result of the high demand for these anticancer and antitumor chemicals, the plant is under severe threat from overexploitation. Currently, *P. hexandrum* (an Indian species) and *P. peltatum* (a North American species) are the primary sources of podophyllotoxin for the pharmaceutical industry. The former is more prolific in its production of podophyllotoxin. *P. hexandrum* plants are being harvested carelessly to meet the increased demand for illicit substances.

P. hexandrum needs around 5 years to mature into a harvestable plant. Because it has been noted that the percentage of resin is at its highest in May, the rhizomes are often picked during this month. May is often a blooming or immature plant fruit stage, preventing fruit and seed development. This could be the main factor causing this plant's population to decline in its native environment. According to Pandey et al. [14], the management of this "Critically endangered status" species might be accomplished by cultivation as a conservation strategy. Only growing *P. hexandrum* vegetatively may result in losing crucial genetic diversity. Because of the dormancy provided by the endosperm and hypocotyls, the seeds exhibit irregular germination and poor seedling establishment. The existence of thick-walled endosperm cells prevents the radical from protruding readily. After acid scarification, seeds are effectively brought out of dormancy by GA3 treatment [15]. Only seeds treated with GA3 didn't germinate adequately [16]. Because GA3 boosts the embryo's development potential and encourages the formation of hydrolytic enzymes (amylase), seeds treated with it in our study had the most significant germination and seedling growth rates compared to seeds treated with IAA and IBA. As a result, this could be an effective technique for regenerating the *P. hexandrum* population.

P. hexandrum can withstand cold, although stress reduces pollen and seed viability. We assessed the same in two altitudinally varied populations. Low-altitude populations had the most pollen and seed viability. Seed germination exhibited the same altitudinal variance. Self-pollination may cause inbreeding depression in long-term snow-covered locations. Early and frequent snowfall on high elevations causes late blooming and withering at immature fruiting [17]. In the current study, it was observed in *Podophyllum hexandrum* that a group of cells initially differentiate from surrounding cells; then, a single archesporium cell develops into a megaspore mother cell. Moreover, micropyle is bitegmic (formed by both integuments). Our studies have also shown differences in the development of female gametophytes in the case of *Podophyllum* from other members of Ranunculales. These observations confirm that *P. hexandrum* is exhibiting a step towards the advancement in the evolutionary process because crassinucellate and endostomic ovules are considered primitive; thus, it will help us understand its systemic position in the family.

CONCLUSION

The study investigated the reproductive features of *Podophyllum hexandrum* Royle, an endangered medicinal plant from Kashmir Himalaya. The female gametophyte development, pollen and seed viability, and seed germination were studied in two populations of *P. hexandrum* from Yaripathri Kulgam and Hirpura Shopian. The *P. hexandrum* ovule was found to be anatropous, bitegmic, and psudocrassinucillate, and the outer integument contributed more to micropyle development, exhibiting steps towards advancement in the evolutionary process. The maximum percentage of pollen and seed viability was

found in populations from low-altitude areas (Yaripathri) compared to high-altitude areas, possibly due to excessive frost conditions. GA3-treated seeds showed the maximum germination percentage in both populations compared to IAA and IBA; thus, this could be the best alternative method for conserving *P. hexandrum* in natural habitats only.

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