Pollination ecology of Himalayan sea buckthorn, *Hippophae rhamnoides* L. (Elaeagnaceae)

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Fruits of *Hippophae rhamnoides* (Elaeagnaceae), commonly known as sea buckthorn or Leh-berry in India, constitute an important source for many medicinal and nutritional products. As the species is of high economic value, attempts are being made to establish commercial plantations for a sustainable supply. There is hardly any information on pollination ecology of this species, which is necessary for establishing and managing commercial plantations. We studied the floral biology and pollination ecology in natural populations of the species growing at Leh-Ladakh. *H. rhamnoides* is dioecious and exclusively wind-pollinated. The male flowers produce an enormous amount of dry, powdery pollen grains that remain viable for up to 48 h. The pollen flow in the air from the source is confined to 15 m. The female flowers are uniovulate. In nature, about 60% of the female flowers get pollinated with an average stigmatic pollen load ($\pm$ SE) of 9.49 ± 0.76. For optimal plantation conditions, it is suggested that a male plant must be inter-planted within 10–15 m of the female plants for effective natural pollination.

Keywords: Dioecy, floral biology, pollination efficiency, sea buckthorn, wind pollination.

POLLEN limitation and pollination failure are the major reasons for suboptimal fruit production in flowering plants. This is particularly prevalent in wind-pollinated and sparsely distributed populations[1–3]. Pollination failure is further aggravated if the wind-pollinated species has separate male and female plants in a population (dioecy).

Hippophae rhamnoides L. (Elaeagnaceae), commonly known as sea buckthorn or Leh-berry in India, is a small, dioecious shrub. The species is distributed in India in the higher altitudinal ranges (3000–5000 m amsl) of Jammu and Kashmir (J&K), Uttarakhand, Sikkim and Arunachal Pradesh[4]. Both wind and biotic pollination (i.e. ambophily) are believed to be present in Elaeagnaceae[5]. Sea buckthorn is one of the few non-leguminous species that fix nitrogen through root nodules[6]. The pulp obtained from the berries has tremendous economical and medicinal value. It is a rich source of carbohydrates, amino acids, essential fatty acids and vitamins A, C, E and K. It also contains carotenoides, flavonoids, organic acids, malic acid, oxalic acid and minerals (Ca, Fe, Mg, Cu, Zn, etc.)[7]. The fruit juice has anti-inflammatory and antimicrobial properties and is used for alleviating pain, in promotion

of tissue regeneration and to boost the immune system. It is also used as an anti-cancer agent and for curing cardiovascular diseases[7,8]. Because of its extensive medicinal use, there have been attempts to initiate commercial cultivation of the species. However, there is hardly any information on its pollination biology which is crucial for establishing plantations and sustaining the yield. Here, we provide details of pollination mechanism and pollination efficiency in sea buckthorn under natural conditions.

The study was conducted during the flowering period (April–May) in 2010 and 2011 at three locations in Leh-Ladakh (J&K): Choglamsar (CV, 34°05.236′N, 077° 36.090′E), Sindhu Darshan (SD, 34°05.269′N, 077° 36.687′E), Water Park (WP, 34°05.236′N, 077°36.090′E). These populations are separated from each other by at least 4 km. The sites of their occurrence are usually windy and receive very low annual precipitation (< 10 cm). The mean annual temperature varies between −40°C and 35°C (ref. 9). The average diurnal temperature during the flowering period ranges between –12°C and 14°C.

H. rhamnoides L. (Elaeagnaceae) also reproduces asexually by means of root suckers and forms massive patches (Figure 1 *a*), often of the same gender. The average distance between the male and female plants in each population ($n = 10$, each population) was determined through line transect method and randomly measuring the distance between the patches of two genders ($n = 10$, each population); the values are expressed as mean $\pm$
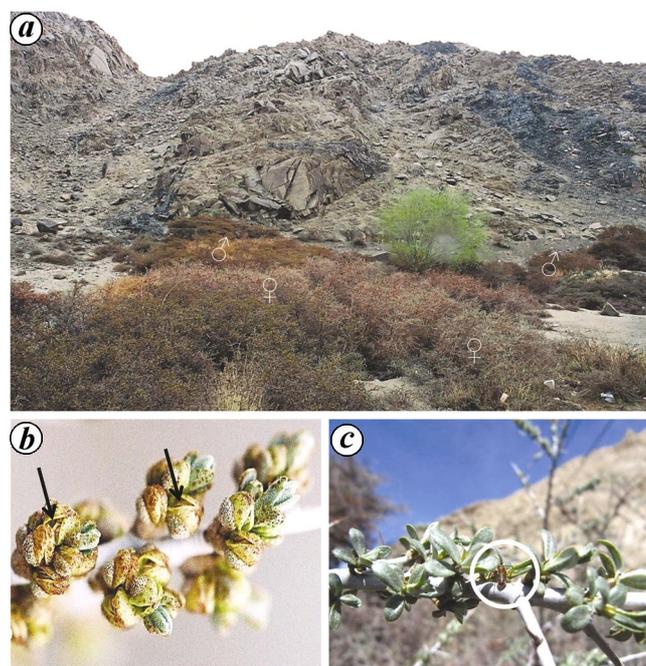

Figure 1. *a*, One of the populations, Sindhu Darshan, at the study site with a massive patch of flowering male (♂) and female (♀) plants of *H. rhamnoides* in bloom. *b*, Part of male flowering twig showing anthesis in male flowers by lateral opening of the perianth lobes (arrows), bar = 2 mm. *c*, Part of female flowering twig with fire ant (encircled) foraging on the female flower.

Table 1. Details of floral developmental stages and stigma receptivity of *H. rhamnoides*

Days before (–) or after (+) anthesis	Key floral feature	Intensity of peroxidases activity*
–3	Stigma enclosed in tepals, green	0
–1	Stigma protruded just beyond the tepals, green	+
0	Stigma protruded beyond subtended bract, erect, yellowish-green	+++
+1	Stigma yellowish-brown	++
+3	Brown colour of stigma	+

*Intensity of bubble emergence; +, Low, ++, Moderate; +++, High.

standard deviation. In CV and SD populations, the average distance between a male and female patch was 4.64 ± 3.8 m (range = 0.5–10 m) and 4.9 ± 3.3 m (range = 0.5–10 m) respectively, whereas in the WP population it was 7.3 ± 2.3 m (range = 5–10 m).

The floral phenology was recorded by monitoring randomly marked male and female plants ($n = 50$ plants each gender, three populations) over a period of 20 flowering days for two seasons. The same set of male plants was monitored for the time of anthesis (opening of flowers). Morphological details of the flowers were studied under a stereo-zoom microscope and the dimensions were recorded with the help of calibrated ocular micrometer.

Each male flower has four anthers and they usually dehisce before anthesis. The suspension method was used to estimate pollen production in a male flower[10]. For this, a ready-to-open male flower ($n = 75$ flowers from five plants, each population) was crushed in 1 ml of suspension solution (50% ethanol with 0.5% Triton-X, v/v) and vortexed for 1 min. Then 25 μl of the suspension was placed over a clean glass slide and a coverslip was gently lowered. The number of pollen grains was counted under the bright field microscope. The value obtained from 25 μl suspension for each flower was multiplied by a factor 40 (1000 μl/25 μl = 40) and the mean total number of pollen grains produced in a flower was computed.

Pollen grains were collected on a glossy sheet by shaking the branches bearing male flowers with freshly dehisced anthers ($n = 10$ male plants from each population). The samples were pooled in cryovials and brought to the laboratory in cool conditions on the same day. These were later used for assessment of pollen viability and longevity. Pollen viability was assessed using fluorochromatic reaction (FCR) test[11], soon after they were brought to the laboratory. The longevity of pollen grains (maintained under laboratory conditions) was estimated by assessing their viability at 24, 48 and 72 h after anther dehiscence. The values were expressed as mean ± SE. The starch grains (reserve material) in the pollen grains were localized by iodine (I_2-KI) test[10]. The duration of stigma receptivity in the female flowers was estimated by peroxidase test[10]. For this, fresh female flowers corresponding to five different developmental stages (Table 1; $n = 5$ flowers for each stage) from each population ($n = 3$) were used.
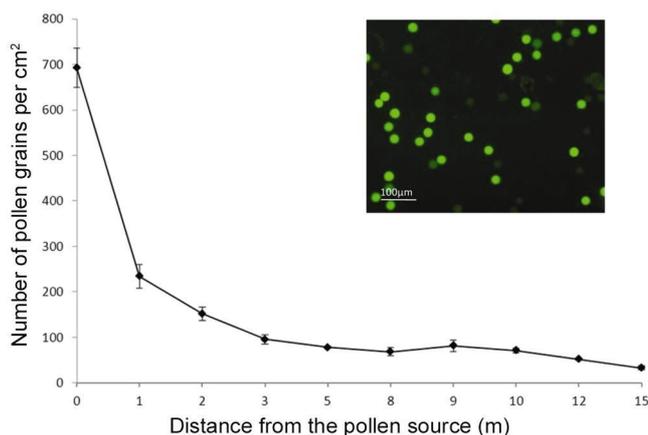

Figure 2. Details of the intra-population pollen flow in *H. rhamnoides*. There is gradual decline in pollen density with increase in the distance from the pollen source. Each point represents the mean ± SE pollen grains per sq. cm of the pooled data from two seasons. (Inset) A fluorescence micrograph of fresh pollen showing brightly fluorescing viable pollen grains; non-fluorescing pollen are not viable.

To assess the possible role of biotic agents in pollination, both male and female plants ($n = 30$ each) were observed at staggered periods (every 2 h for a total of 7 observations of 30 min each) from 0700 to 2100 h in each population for 10 consecutive days during the peak duration of flowering. Insects visiting the female and male flowers were collected separately in glass vials containing cotton soaked in ethyl acetate. The insects were brought to the laboratory and observed under the bright field microscope for pollen load.

To ascertain the role of wind in pollination, sticky-slides method was employed[12]. Four or five 1 cm² grids were marked on the reverse side of the glass slides (size: 1 × 3 inches) with the help of a black permanent marker. The slides were smeared with petroleum jelly and exposed vertically (2–3 ft above the ground) at different distances (0, 1, 2, 3, 5, 8, 9, 10, 12, 15, 18, 20 m) from the isolated male patches (Figure 2). Three or four slides were exposed between 0800 and 0830 h and collected after 24 h from the field. The experiment was conducted twice in a season for two consecutive days and the data were obtained from a total of 180 slides during the study period (2010–11). The number of pollen grains present within the 1 cm² grids was counted (in each slide) under

the microscope. The data were pooled for each of the distances, as the difference in pollen count between slides exposed at the same distance was not significant between years (independent t-test, $P < 0.05$; the t-values for each of the distances mentioned above were $t_{(14)} = -0.12$; $t_{(14)} = -0.10$, $t_{(14)} = -0.02$, $t_{(13)} = 0.26$, $t_{(13)} = -0.86$, $t_{(12)} = -0.32$, $t_{(11)} = -0.15$, $t_{(11)} = 0.80$, $t_{(13)} = 0.22$ and $t_{(23)} = 0.95$ respectively).

To compute the natural pollination efficiency (i.e. amount of pollen load on open-pollinated stigmas), the freshly anthesized female flowers ($n = 10$ flowers) on randomly selected plants ($n = 5$ plants) in each population ($n = 3$ populations) were tagged and allowed for natural pollination. After 72 h these flowers were fixed over cotton soaked in FPA solution (37% formaldehyde : propionic acid: ethanol: double distilled water in 10 : 5 : 50 : 35, v/v). The stigma was dissected in a drop of auramine-O' solution (0.01% auramine-O' in 0.05 M Tris/HCl buffer, pH 7.2, EX-460 nm; EM-550 nm) and the total number of pollen grains was counted under an epifluorescence microscope (Eclipse 80i Nikon, Japan)[13]. After ascertaining the normal distribution, the difference in natural pollination efficiency (dependent variable) among the three populations was analysed through nested one-way ANOVA; plants and populations were considered as random and fixed factors respectively (SPSS 16 software, 2007, Chicago Inc.).

Flowering in *H. rhamnoides* occurred between mid-April and the first week of May. The plants are completely leafless at the time of flowering (Figure 1 *a*). The male flowers are borne in condensed axillary and terminal racemes (Figure 1 *b*). The male flowers are small (3.4 ± 0.1 mm in length) and each is represented by four stamens enclosed in two perianth lobes. During anthesis (0800–1200 h), the perianth lobes open along their margins while their apices remain united (Figure 1 *b*). It takes 2–3 days for the male flowers to dispense all the pollen grains. The amount of pollen produced is large ($81,714.71 \pm 1894$/flower). The pollen grains are small (26.47 ± 0.37 μm), dry and contain starch grains as reserve material. The viability of fresh pollen grains was $57.71 \pm 2.26\%$ (Figure 2), which declined to $46.08 \pm 3.83\%$ and $31.27 \pm 2.76\%$ after 24 and 48 h of anther dehiscence respectively. Pollen grains lost viability completely by 72 h after anther dehiscence.

The female flower is represented by a gynoecium covered with two partly fused perianth lobes. During anthesis (0900–1200 h), a shining yellowish-green expanded stigma emerges from the perianth lobes. The stigma is most receptive at the time of anthesis and the receptivity gradually declines and becomes negligible after three days of anthesis (Table 1). Stigma is non-papillate, scorbiculate and dry type[14]. At anthesis, the ventral receptive side of the stigma faces the air and dorsal side faces the subtending bract. Each flower produces only one ovule.

At the population level, anthesis in male plants precedes the female plants by 2–3 days. Also during each day of flowering, the male flowers invariably anthesize nearly 1 h earlier than the females. The flowers lack nectar and fragrance.

Coccinella septempunctata (lady bird beetle) and *Thrips* spp. were observed exclusively within the male inflorescences and fire ants (*Solenopsis* sp., Figure 1 *c*) amidst the female inflorescences. All the visits by the fire ants to the female flowers were confined between 0700–1600 h. Fire ants collected from the female flowers did not show pollen load on their body parts and usually caused damage to the stigmas at anthesis. The insects associated with male flowers were observed to forage for starchy pollen grains. Many bird species such as *Chaimorrornis leucocephalus* (White capped redstart), *Pica pica* (magpie), *Prunella rubeculoides* (Robin accentor), *Phylloscopus affinis* (Tickell's leaf warbler) and *Saxicola insignis* (Hodgson's bushchat) visited both the male and female plants. But none of these birds came in contact with the flowers; they used the dense canopies mainly for nesting.

Pollen flow showed a leptokurtic distribution (Figure 2). The density of airborne pollen grains was highest in those female patches (692.5 ± 43 cm^{-2}) which were nearest to male patches (i.e. at 0 m) and the density declined with distance. The flow of pollen was recorded up to a distance of 15 m from the male patches, with an average pollen density of 33.6 ± 3.73 cm^{-2} (Figure 2). Slides kept beyond 15 m from the source lacked pollen deposition.

Nearly 62% of the open-pollinated stigmas showed pollen deposition. The stigmatic pollen load ranged between 2 and 31, and there was a significant variation in pollination efficiency among the three populations (Table 2). However, plants within the population did not exhibit any variation (Table 2). The mean pollen load ($\pm$ SE) was 14.72 ± 1.09, 8.64 ± 0.58 and 5.12 ± 0.35 per stigma in CV, SD and WP populations respectively (Figure 3).

One of the obvious outcomes of dioecy in plants is that it ensures outbreeding[15,16]. Dioecious species are generally pollinated by wind and sometimes by unspecialized biotic pollinators[15]. This correlate has been aptly validated in a recent phylogenetic analysis which demonstrated that plant lineages with unisexual flowers are predominantly associated with wind pollination[17].

Table 2. Nested one-way analysis of variance of pollination efficiency (stigmatic pollen load, dependent variable) in plants (random factor) among the three populations (fixed factor) of *H. rhamnoides*

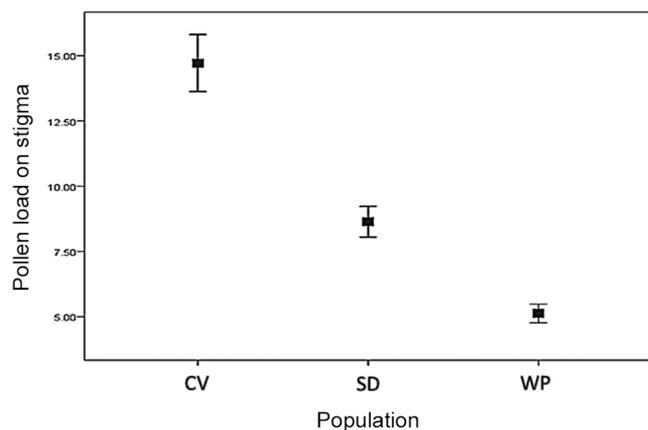| Effect	df	ss	F	P
Populations	2	2304.0	21.107	0.000
Plants (populations)	12	382.0	0.583	0.852
Residual	135	7367.9		

Figure 3. Graph showing natural pollination efficiency (pollen load on stigma) among the three populations (CV: Choglamsar, SD: Sindhu Darshan, WP: Water Park). Each point represents the mean ± SE of pollen load on stigma in each population.

The present study clearly shows that wind is the exclusive mode of pollination in *H. rhamnoides*. Both the floral features and the prevailing ecological conditions at the study site profoundly favour the occurrence of wind pollination in the species. Open habitat with moderate to high wind speed and low precipitation are features favourable to effect wind pollination[5,18]. Features such as gregarious flowering in leafless and open canopies, condensed male and female inflorescences, copious pollen production and small and dry pollen grains with smooth exine surface are considered effective in maximizing the airborne dispersal of pollen grains[5,18].

Although some insects were recorded on the male and female flowers, their visits were exclusive to a particular gender of the plant and they did not bring about pollination. It is quite likely that a pollen forager capable of visiting both the types of flowers could be missing from the region. Thus, wind acts as the primary mode of pollination in the species. This observation in turn supports the hypothesis that reliance on biotic pollinators in dioecious clades could make them more vulnerable to extinction in temperate regions because they are more susceptible to fluctuations in biotic pollinators[19].

The pattern of anthesis and temporal variations in anthesis of flowers in male and female plants of a dioecious species (prior anthesis in males) may have fitness consequences[20,21]. The pattern of anthesis in male flowers as observed in the three populations of *H. rhamnoides* was the same. In sea buckthorn, during anthesis in male flowers the two perianth lobes remain apically fused while their margins open up on either side to expose the dehisced anthers. The pollen grains are dispersed whenever wind flushes through the channel made by the two lateral openings. This mechanism of pollen dispersal appears to be advantageous to the species, as it ensures a continuous availability of airborne pollen grains in small batches.

Usually the wind-pollinated plants exhibit low pollination efficiency (stigmatic pollen load) in spite of enor-mous pollen production[17,18]. This is largely because pollen deposition through wind is not targeted on the conspecifics and an enormous amount of pollen is wasted[22,23]. On the contrary, in *H. rhamnoides* ~ 60% of the flowers showed pollen deposition (range = 2 to 31 pollen per stigma). This range of pollen deposition in an appreciable proportion of flowers is a fairly better representation among the wind-pollinated species and could be attributed to early flowering in male plants, prior anthesis in male flowers (an hour before female anthesis) and a distinct pattern of dispatch of pollen grains. Prior flowering in male plants and anthesis in male flowers ensure the availability of airborne pollen grains when the female flowers are receptive[20,21,24]. The lowest pollination efficiency from WP population (with greatest inter-gender plant distance) and in general, the absence of pollen deposition in nearly 40% of the flowers could be attributed to distance between female and male plants beyond the effective range of pollen flow in the species, wind direction and pollen loss usually associated with the wind-assisted pollen dispersal mechanism.

In wind-pollinated species, pollen dispersal usually follows a leptokurtic distribution curve and the effective distance of pollen flow among the populations is influenced by spatial segregation, relative sizes and densities of conspecifics, phenology and intervening vegetation[25]. Under wild conditions, male and female plants of sea buckthorn form dense patches, which may cause physical hindrance to distant flow of pollen grains even when the plants flower *en masse*[18,26,27]. Besides the occurrence of clonality due to formation of root suckers, a short distance of pollen flow observed in the sea buckthorn could also be one of the reasons for low intra-population and high inter-population genetic diversity as observed in an earlier study[4]. Short-distance pollen flow has significant implications for propagating commercial plantations of *H. rhamnoides*. It is recommended that the mean distance between the female and male plants in *H. rhamnoides* plantations be within the 10–15 m range for adequate pollination.

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Impact of climate change on the flowering of *Rhododendron arboreum* in central Himalaya, India

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Studies from different parts of the world have generated evidences of the effects of climate change on phenology and persistence of species. However, datasets or evidences are lacking for majority of the regions and species, including the climate-sensitive Himalayan biodiversity hotspot. Recognizing this gap in the information and realizing wide-ranging implications of such datasets, the present study generates evidences of changes in flowering phenology of an important trees species, *Rhododendron arboreum* in Indian central Himalaya. Real-time field observations (2009–2011) showed peak flowering during early February to mid-March. Analysis on long-term temperature data revealed significant ($P < 0.01$) increase in seasonal (winter and post-monsoon) and annual mean maximum temperature. Generalized additive model (GAM) using real-time field observations (2009–2011) and herbarium records (1893–2003) predicted 88–97 days early flowering over the last 100 years. Furthermore, GAM using long-term temperature data, real-time field observations and herbarium records depicted annual mean maximum temperature responsible for shifts in flowering dates of the target species. The study provides an important insight of species response to climate change in the Indian central Himalaya and highlights the need for further research on the subject to improve our understanding of the effects of climate change on species and consequently on ecology of the region.

Keywords: Climate change, flowering phenology, herbarium records, *Rhododendron arboreum*.

THE phenological responses of plants, particularly the early flowering ones, are considered among the prominent biological indicators of climate change[1,2]. In this respect, various studies from different parts of the world have provided convincing evidences[3–7]. However, most of these studies have relied on long-term datasets, created for the specific purpose of phenological measurements[5]. Unfortunately, for many regions and species such datasets/documentation are/is often not available[6]. Therefore, the need to have new source of data to build a more complete global picture is evident.

The Indian Himalayan Region (IHR), recognized amongst 34 global biodiversity hotspots (Conservation