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RESEARCH ARTICLE

Genetic System of *Artemisia maritima* L.: An Overexploited Medicinal Species Under Stress

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Abstract *Artemisia maritima* L., a potent source of Santonin (a drug used to expel roundworms), forms isolated populations in Kishtwar area (1760 m asl) of J&K state, India, in north-west Himalayas. Once a luxuriantly growing plant, the species has seen a decline in its distribution in the recent past in the area of study. While the overexploitation is an obvious reason for the same, the authors studied in detail its meiotic and breeding system, to identify intrinsic factors, if any. The plant is a stable diploid ($2n = 18$) and is predominantly outcrossed. It exhibits both anemophily as well as entomophily. Seed set on open pollination is adequate ($78.38 \pm 2.28\%$) in dense populations ($n = 220$). In case the outcrossing fails, the species keeps provision for selfing also, a feature rare in genus *Artemisia* L. However, selfing results in extremely low fruit set (20.8%) depicting inbreeding depression. Enforced selfing can thus be cited as one of the major intrinsic factors reducing the propagation of this species.

Keywords *Artemisia maritima* · Anemophily · Entomophily · Inbreeding depression · Selfing

Significant statement Intrinsic factor contributing to dwindling populations of *A. maritima* is inbreeding depression. Individual plant of *A. maritima* produces good quantity of seeds on open pollination. On geitonogamous pollination/autogamy, seed set is reduced drastically. A species with efficient contrivances for outcrossing is supposedly forced to self on account of decline in population size due to anthropogenic stresses. It is thus facing the intrinsic challenge of inbreeding depression.

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Introduction

Artemisia maritima L. a member of family Asteraceae has immense medicinal virtues; the species is reported to have aromatic importance too [1–3]. A perennial temperate species, *A. maritima,* is reported from only some areas of north-western India including Kashmir, Kurram, Kishtwar and Gurez (1580–1760 m asl) as compared to several other *Artemisia* species (*A. nilagirica* and *A. scoparia*) of the area that enjoy a wide distribution at different altitudes (332–1705 m asl) [4, 5]. The area of present study is Kishtwar located at an altitude of 1760 m asl in north-west Himalayas. The species once used to grow well in dense natural strands in this area. The populations have squeezed considerably at present, while some anthropogenic stresses are vivid at some locations in terms of overexploitation, construction of roads and dams. The present study was conducted to look for internal threats, if any, reporting the genetic system of the species in detail for the first time.

Material and Methods

Plants were tagged in the field and studied for gross and floral morphology. Floral structure with emphasis on the reproductive apparatus including pistil length, size of ovary and length of stamens was studied in the laboratory from the flowers collected at random from the field. Field

surveys were conducted regularly (weekly in initial stages to constant stay in the area for 15 days during full bloom) to monitor the pollination and breeding system of the species.

Pollen Stainability and Viability

Pollen stainability and viability were checked by two tests: stainability in 1% acetocarmine and enzyme assay by 2,3,5-triphenyl tetrazoliumtrichloride [6].

Stainability Test

Mature but undehisced anthers were squashed in 1% acetocarmine. All deeply stained pollen grains were considered stainable.

Enzyme Assay Test (FDA Test)

Pollen grains from ready to dehisce anthers were squashed in a drop of saturated solution of fluorescein diacetate in acetone and observed under fluorescence microscope. Pollen grains that fluoresce in UV-light were considered as viable, and those that were deformed and did not fluoresce were considered as non-viable.

Pollen Ovule Ratio

Pollen count per flower was estimated by calculating the number of pollen grains per anther just prior to dehiscence by squashing it in a drop of 1% acetocarmine. Ovules were counted by carefully dissecting out the individual ovaries with the help of fine and sharp needles. Pollen ovule ratio was calculated by dividing the pollen count per flower with the number of ovules in the same flower.

Anthesis and Anther Dehiscence and Stigma Receptivity

Anthesis as well as anther dehiscence was observed at regular intervals of time from tagged plants in the field. Time taken by an individual flower and the whole inflorescence to open was recorded. For checking the anther dehiscence, pollen shedding and stigma receptivity, flowers were monitored at different timings before and after anthesis. Stigma receptivity was checked as per Koul et al. [7].

Pollination Studies

For confirming the role of wind in pollination, hanging slide method was employed. Glass slides (7 × 2.5 cm) smeared with a mixture of glycerine and egg albumin (in the ratio 1:1) were suspended from T-shaped wooden stands (almost of the same height as the plants) fixed around the plants at a distance varying between 1 and 2 m from the pollen source during blooming period of plants. These slides were left exposed for 24 h and examined microscopically thereafter for the number of pollen grains trapped.

Plants were also checked for the nature of attractants and reward in their floral and extrafloral parts and for the presence and frequency of biotic pollinators on them. Pollinator visitation was assessed in the field by observing the plants and inflorescences throughout the blooming period. The visitors were scrutinized for pollen load on their body parts.

Fruit and Seed Set

Fruit and seed set were observed in the plants growing open in the field. Few inflorescences were also bagged to check the fruit/seed set on forced selfing in this species. Number of flowers/capitulum and the number of fruits set/capitulum were counted for these plants. Percentage fruit/seed set was calculated as following.

$$\text{Percentage fruit/seed set} = \frac{\text{Fruit count/inflorescence}}{\text{Flower count of the same inflorescence}} \times 100$$

Cytological Studies

Meiosis

Pollen mother cell meiosis was studied from young immature flower buds fixed during morning hours in a mixture of 3 parts of ethyl alcohol and 1 part of acetic acid. After fixation for 24 h, the buds were washed in water and preserved in 70% ethyl alcohol at 4–6 °C. Finally, the anthers were squashed in 1% propiocarmine.

Karyotype Preparation

Somatic chromosome spreads were obtained from root tips of germinating seeds. The tips were pretreated with paradichlorobenzene solution for 3.5 h. These were washed and preserved in 70% alcohol. The fixed root tips were washed and hydrolyzed in 9:1 mixture of 1% aceto-orcein and 1 N HCl for 13 min in an oven maintained at 60 °C. These were then squashed in 1% aceto-orcein on a clean glass slide. All photomicrography of chromosomal preparations was done using unit-Nikon ECLIPSE E 400 attached to a digital colour camera SAMSUNG SDS-312. The field photography was done with the camera SONY DSC-H10. The bar on each photomicrograph represents 10 μm.

Results and Discussion

Plant and Floral Morphology

Plants of *Artemisia maritima* are perennial xerophytic shrubs (Fig. 1a). These perennate in winter through rootstock that sprouts in the month of March–April to produce aerial offshoots. Each rootstock produces 5–13 offshoots each year. Individual offshoot attains a height of 72.31 ± 1.05 cm with number of branches per offshoot varying between 14 and 63. Species enters into reproductive phase after 5–6 months of vigorous vegetative growth. Flowering initiates in early October; temperature during flowering period ranges from 5.6 to 26.5 °C, and relative humidity averages 64.77%. Flowers are borne on axillary spikes terminating into heads. The small oblong homogamous floral heads are yellowish green in colour and are arranged in racemes which are either drooping or erect. Size of individual floral head averages 0.32 ± 0.02 cm. The discoid capitula are homogamous, being composed of 7–12 homomorphic tubular florets (Fig. 1b). Florets are bisexual, regular, actinomorphic, pentamerous and epigynous. Each floret bears five petals united to form a short cylindrical corolla tube that merges into a narrow campanulate limb. Reproductive apparatus comprises of five syngenesious stamens and a pistil. Dithecous, basifixed and introrse anthers are fused to form a hollow cylinder which surrounds the style. Length of individual stamen averages 0.2 cm. Long bristles are present at the tips of anthers. The female reproductive apparatus comprises of a pistil 0.38 ± 0.03 cm long on maturity. Pistil consists of a bicarpellary, syncarpous, inferior and unilocular, one-ovuled ovary with basal placentation, and ovary averages 830 μm in size. A mature style is elongated and terminates into bifid stigma. Stigma is dry, papillate and yellowish green. The two stigmatic lobes remain appressed to each other inside the floral tube. At maturity, the lobes of the stigma curve outwards exposing the inner receptive surface. An epigynous nectar secreting disc is present at the base of the corolla tube surrounding the style.

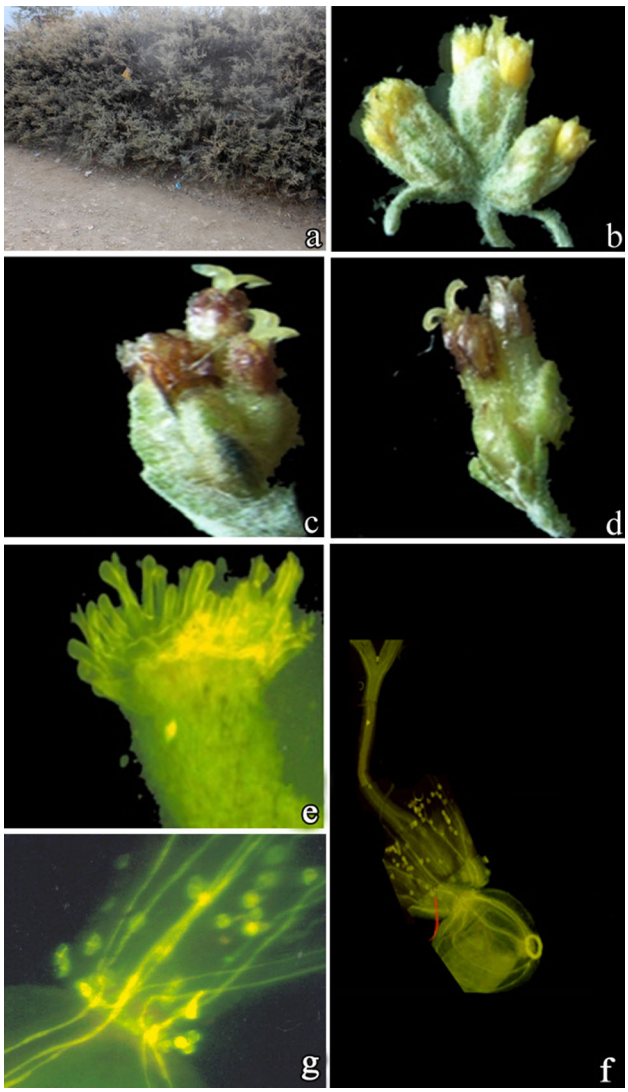

Fig. 1 *Artemisia maritima* L. **a** Plants growing in natural habitat, **b**–**d** showing florets at various stages of development. Note the anther tube and stigmatic development. Fluorescence microphotographs showing, **e** pollen grains germinating on the stigmatic surface, **f** germinating pollen at the nectary, **g** pollen tube pathway from nectary to ovary

Floral Biology and Pollination Mechanism

The florets are protandrous; dehiscence of anthers takes place a day before the opening of the florets. Dehiscence initiates by the formation of longitudinal slits in the anthers and proceeds simultaneously in all the anthers. Dehiscence is introrse, and pollen is shed inside the staminal tube formed by the anthers. As the floret opens, the anther tube is exerted fully out of the floral tube by rapid elongation of filaments leaving the pollen inside the floral tube. The pollen is pushed outwards slowly with the growth of the style. As the pollen is shed, style as well as the stigma with closed stigmatic lobes is quite short (Fig. 1b). It elongates later, and after 1–2 days of floret opening it grows and pushes pollen out of the floral tube into anther cylinder and later outside (Fig. 1c, d). The growing styles thus act as a device and help in presenting the pollen to the surface of the floral tube. As the pollen is pushed outside, it gets trapped in the long bristles present at the tips of exerted anthers. These bristles are sterile anther apices which act as pollen holders. By the time the bristles get loaded with yellow powdery pollen, stigma with its appressed lobes is still seen emerging out of the floret and then through the tube formed by anthers which are now empty and non-functional. Stigma in the species is dry and papillate

(Fig. 1e). It emerges out and spreads its lobes and becomes receptive 4–5 days after the self-pollen has been drifted off by wind and/or by insects.

Plant disposes its pollen mainly by wind; this was confirmed by hanging slides smeared with Meyer's albumen around blooming plants in the field. About 48–83 pollen grains were observed per slide after 24 h of exposure. The plant is also entomophilous and is visited by honeybees (*Apis mellifera*) and butterflies in the blooming period, the former being the most frequent visitor. Honeybees carry ample pollen load of the species on their body with maximum number on hind and middle legs indicating their role in pollination of this species. Average pollen carried per insect was 837.3 (636–1284). Time taken by inflorescence to complete anthesis varies between 23 and 28 days (25 ± 0.04). Since all the inflorescences in a plant bloom more or less simultaneously, whole plant completes its anthesis in an average of 43 ± 0.6 days.

Dual Pollen Germination

Although the elongation of style pushes large quantity of pollen outside each floret, some amount remains sticking to the inner surface of petals and on apical region of ovary on epigynous nectary. As a deviation from normal germination of pollen on the stigmatic surface, in this species some pollen grains were seen germinating on the nectary. Pollen tubes emanating from the pollen germinating on nectary, capping the ovary, were observed entering the ovary (Fig. 1f, g). The species thus represents an example of dual pollen germination, i.e. on nectary capping the ovary and on the stigmatic surface [8].

Pollen Output, Stainability and Viability

Pollen output/floret averages 5244 ± 6.21 (4026–5938). Pollen grains are smooth-walled, spheroidal, aperturate (triporate), and average size of pollen grain is 20 ± 1.2 μm. Percentage stainability calculated by using 1% acetocarmine averages 96.39%. The viability as tested by using 2,3,5-triphenyl tetrazolium chloride averages 92%.

Reproductive Output

Fruiting in *A. maritima* initiates during early November and continues till early December. Fruit is cypsela; a dry indehiscent one-seeded fruit developed from a bicarpellary, syncarpous, inferior, unilocular ovary. Each fruit remains enclosed by persistant bracts. Seed, one per fruit bears a straight embryo, is brown or grey in colour and small in size. Fruit and seed set on open pollination in dense populations are very high and average 78.38 ± 2.28%. Out of these, 75.29 ± 2.36% seeds are healthy. On bagging, % healthy seed set declines drastically and averages 20.8 ± 1.47%.

In twenty-five individuals, single inflorescence was selected per individual at random, and in all the flowers of these inflorescences, stigmas were decapitated as soon as they came out of the florets, i.e. before these became receptive. The same was done to estimate seed set via ovarian pollination. The data on fruit set were recorded for these inflorescences; it averaged 21.09 ± 2.18. Each fruit contained single seed. Except for few seeds which were shrivelled and curved, all seeds resembled the ones, set in open pollinated flowers (% age Healthy = 19%).

Cytology

Meiosis

Plants were scanned for meiotic details. A total of 240 cells were observed at different stages of meiosis in these plants, 52 cells (21.66%) at diplotene (Fig. 2a), 46 cells (19.16%)

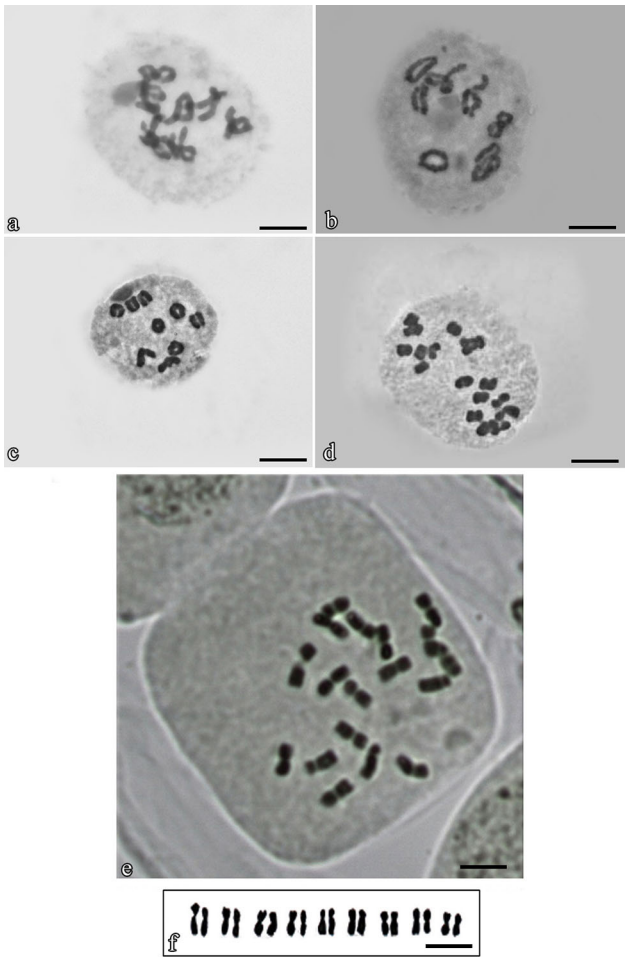

Fig. 2 Pollen mother cells of *A. maritima* L. at **a** Diplotene. **b** Diakinesis. **c** Metaphase-I. **d** Anaphase-I. **e–f** Somatic metaphase spread and karyoidiogram of *A. maritima* L

at diakinesis (Fig. 2b) and 90 cells (37.5%) at metaphase-I (Fig. 2c), among these showed the presence of nine bivalents revealing the species to be diploid with $2n = 18$. Chiasmata frequency per pmc at diplotene averages 12.5; thus, the recombination index in the species is 21.5. Fifty-two cells scanned at anaphase-I revealed normal segregation of chromosomes with 9:9 distribution at each pole in all the cells scanned (Fig. 2d).

Karyotype Analysis

Metaphase spreads obtained by squashing root tips in 1% propiocarmine revealed the presence of 18 chromosomes that form nine pairs (Fig. 2e, f). The karyotype formula for the plants is 2M + 16SM. On the basis of inflorescence and floral characters as elaborated above, *A. maritima* is placed in section Seriphidium of tribe Anthemideae of family Asteraceae, which is the only section in the tribe characterized by the presence of homogamous capitula comprising of all hermaphrodite florets. Rest of the subgenera are either heterogamous (Absinthium, Abrotanum) or functionally monoecious (Dracunculus). Twenty species of *Artemisia* have been reported from north-west Himalayan Indian state of Jammu and Kashmir where the present study has been conducted. A study on eight species from this lot by the authors reveals that *A. maritima* is the only homogamous species. Rest are either gynomonoecious (*A. nilagirica*, *A. tournefortiana*, *A. sieversiana* and *A. gmelini*) or functionally monoecious (*A. glauca* and *A. scoparia*) [9, 10].

Florets in *A. maritima* are strongly protandrous, and the anther dehiscence gets initiated one day before the opening of the corolla tube. Stigma receptivity on the other hand initiates 3–4 days after the floret opens. This creates a gap of 4–5 days between the two. This sequence of events is followed in all the species of *Artemisia* studied by the authors, with only difference being the number of days spanning between anther dehiscence and stigma receptivity [9, 11, 12]. Strong protandry combined with pollen presentation mechanism creates conditions conducive for cross-pollination in *A. maritima*. Pollen pushed outside the floral tube is efficiently dispersed by both wind as well as insects making this species an ambophilous one, a rare feature again [13–15]. A unique feature of this species unlike any other *Artemisia* species reported so far is its capability to set some healthy seed by selfing also.

In the analysis of eight species of the genus inhabiting north-west Himalayas, the authors report that healthy seed set via selfing is negligible [9, 10, 16–18]. To the best of the knowledge of the authors, this is the only species of *Artemisia* L. setting moderate amount of healthy seed by selfing also [19]. Interesting phenomenon of dual pollen germination is shown by the species, i.e. germination of pollen grains on nectary capping the ovary wall in addition to the stigma [8] which gives chance to the self-pollen to affect fertilization. Only 21.09% seed is set in this germination and 20.8% on forced selfing revealing that inbreeding is injurious to the species.

Meiotic system reveals the species to be a perfect diploid with 18 chromosomes forming nine bivalents at diakinesis and metaphase-I. Normal segregation of chromosomes and high pollen viability point towards the cytologically stable nature of the species in the area of study. Elsewhere, this species has been reported to be cytologically flexible also with several intraspecific chromosomal races mainly diploid ($2n = 18$), tetraploid ($2n = 36$) and hexaploid ($2n = 54$) [20, 21]. In another report from Kashmir region of India, the species has been reported to be diploid with $2n = 18$ [22]. An intrinsic factor that may be contributing to the decline of this cytologically stable race in the area of study according to the authors is imposed selfing.

Conclusion

An intrinsic factor contributing to dwindling populations of *A. maritima* sprawling in India is most probably inbreeding depression. Species is cytologically stable and is a diploid race in north-west Himalayas. It produces viable pollen that is dispersed efficiently. The plant has spreading habit, and the single genotype covers quite a good area with 5–13 highly branched offshoots. With the habitat destruction and overexploitation taking place, the number of individuals with distinct genotype coexisting together is coming down and thus some seed production is mainly through geitonogamous pollination. In spite of the fact that individual plant of *A. maritima* produces good quantity of seeds every year, proportion of healthy seeds is going low. Species equipped with a stable meiotic system and efficient contrivances for outcrossing (strong protandry and abmophily) is unable to generate enough genetic variation when it undergoes selfing for seed set.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest regarding the publication of this manuscript.

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