High Pollen Sterility and 2n Pollen Grains in an Asynaptic 4x Cytotype (2n=48) of Solanum nigrum L.

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Summary Cytological investigations have been carried out on the 9 wild accessions of Solanum nigrum L., a medicinally important species having wide-range therapeutic properties. Male meiotic studies performed in 9 accessions revealed the presence of 3 intraspecific cytotypes, 2x (n=12), 4x (n=24) and 6x (n=36) in the species. The course of meiosis in 8 of the 9 accessions among the 2x, 4x, 6x cytotypes was noticed to be perfectly normal resulting in very high pollen fertility (95–100%). However, 1 accession of 4x cytotype scored from Palchan (Solang Valley, Kullu, Himachal Pradesh) depicted irregular chromosomal behaviour during meiosis due to the presence of all 48 chromosomes as univalents. The further course of meiosis in this individual was highly irregular with the presence of large number of lagging and unoriented chromosomes, eccentric positioned univalents in the PMCs and unequal distribution of chromosomes at anaphase-I. Microsporogenesis was also abnormal which is characterized by the presence of irregular sporads, such as dyads, triads and polyads. Lagging chromosomes constituted micronuclei at sporad stages. Consequent to all these meiotic irregularities, 95% of the pollen grains were observed to be sterile. Pollen grains were also recorded to be of different sizes. In meiotically abnormal 4x accession, pollen grains were of 2 different types compared to normal 4x plants showing perfectly normal meiosis. The large pollen grains, which were measured to be 1.25–1.5 times bigger than normal sized pollen grains, were considered as unreduced or 2n pollen grains. The role of such 2n (unreduced) pollen grains in producing polyploid genotypes through sexual polyploidization in a chromosomally variable S. nigrum (2x, 3x, 4x, 5x, 6x, 8x, 9x, 12x) has also been discussed.

Key words: Solanum nigrum, Cytotype, Pollen grains, Univalents, Meiosis, Parvati Valley.

Solanum nigrum L., popularly referred as black nightshade or Makoi, is native to Eurasia and a very common and short-lived perennial herb which usually grows as a weed in diverse habitats between altitudes of 600–3000 m. It can be cultivated in tropical and subtropical agro climatic regions. The species is of immense medicinal value and has been extensively used in Oriental medicinal systems to treat ailments such as fever, inflammations and pain (Acharya and Pokhrel 2006, Zakaria et al. 2006, Jain et al. 2011). The species has also been used as an antioxidant, anti-tumorigenic, diuretic, hepatoprotective, and antipyretic agent (Lee and Lim 2003, Raju et al. 2003, Jain et al. 2011). It has a number of ethnobotanical uses (Qureshi et al. 2009, Egunyomi et al. 2010), for example the tea of tender leaves is used for curing flu, cough and fever. Leaves are also used as mosquito repellent. Dried fruits are used for stomach diseases. It is used cooked as pot herb and used as an anti-inflammatory for the internal organs. A poultice of leaves is applied on wounds and burnt skins.

Besides its wide-ranging therapeutic properties, the species is reported to show phenotypic
variation, particularly in its vegetative features such as plant habit, leaf size and form, fruit colour and stem winging (Edmonds and Chweya 1997). Also in terms of chromosome number, the species had been found to be very variable and an array of chromosome numbers \(2n=24, 36, 42, 48, 54, 56, 60, 63, 64, 72, 108, 144\), c.f. Indexes to Plant Chromosomes Numbers, Darlington and Wylie 1955, Fedorov, 1969, Ornduff 1968, 1969, Moore 1970, 1971, 1972, 1973, 1974, 1977, Löve and Löve 1974, 1975, Goldblatt 1981, 1984, 1985, 1988, Kumar and Subramanian 1986, Goldblatt and Johnson 1990, 1991, 1994, 1996, 1998, 2000, 2003, 2006, Khatoon and Ali 1993) are reported from India and outside of India. It seems that the species has exploited both polyploidy (2\(x\), 3\(x\), 4\(x\), 5\(x\), 6\(x\), 8\(x\), 9\(x\), 12\(x\)) and aneuploidy (2\(n=42, 48, 54, 56, 60, 63, 64\)) for its evolution and in order to adapt to diverse habitats. Out of the 8 known intraspecific cytotypes the most commonly found cytotypes are diploid (2\(n=24\)), tetraploid (2\(n=48\)) and hexaploid (2\(n=72\)).

During the course of the present study of male meiosis in the species from the cold regions of Northwest Himalayas, 3 cytotypes, the diploid (\(n=12\)), the tetraploid (\(n=24\)), and the hexaploid (\(n=36\)) have been found. Surprisingly 1 individual of the 4\(x\) cytotype collected from Palchan village (Solang Valley, Kullu, Himachal Pradesh) showed very high pollen sterility while the other individuals of 4\(x\) and 2\(x\) and 6\(x\) cytotypes showed nearly 100% pollen fertility.

The aim of the present investigation was to study the cause of high pollen sterility in the plant of 4\(x\) cytotype and to correlate it with cytolological behaviour. The aim was also to compare the chromosomal behaviour during course of meiosis, microsporogenesis and pollen fertility in all 3 cytotypes.

Materials and methods

**Study site, plant material and its identification**

Materials for male meiotic studies were collected from the wild plants growing in 9 different localities in the Kullu district of Himachal Pradesh (Table 1) in June–August of 2010–2011. The plants investigated for male meiosis were identified using regional floras and compared with the specimens deposited at the Herbarium of Botanical Survey of India, Northern Circle, Dehra Dun. The voucher specimens were deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN) and the accession numbers obtained for each plant under different cytotypes are given in Table 1.

**Fixation of plant material, chromosome counts, meiotic course and sporad analysis**

For meiotic chromosome counts, unopened floral buds of suitable sizes were fixed in a freshly prepared Carnoy’s fixative (6:3:1 of alcohol: chloroform: glacial acetic acid) for 24 h at room temperature. The material was subsequently transferred to 70% alcohol and stored in a refrigerator until analyzed. Meiocytes were prepared by squashing the developing anthers, and staining them with acetocarmine (1%). Chromosome number was determined at diakinesis, metaphase-I (M-I) and anaphases-I (A-I) from the freshly prepared slides with a light microscope Olympus. 300–500 pollen mother cells (PMCs) were analyzed for meiotic behaviour at different stages, early prophase-I, metaphase-I/II (M-I/II), anaphase-I/II (A-I/II), telophase-I/II (T-I/II). To observe the effect of meiotic irregularities after T-II on microspore formation, sporad analysis was also carried out.

**Estimation of pollen fertility and pollen grain size**

For testing the pollen fertility, a stainability test was used in which anthers from a mature flower bud were crushed (with the help of a glass rod) in a drop of glycerol–acetocarmine (1:1) mixture and aniline blue (1%) and left it for 2–3 h at room temperature to obtain a proper stain. Up to 200–500 pollen grains were studied for pollen fertility and size frequencies. Well-filled pollen grains with stained nuclei were taken as apparently fertile, while flaccid unstained/poorly stained
pollen grains were counted as sterile. In each case, the size of 50 pollen grains was measured using an occulomicrometre.

**Photomicrographs**

Photomicrographs from the freshly prepared desirable slides having clear chromosome counts, dyads, triads, tetrads, polyads and pollen grains were taken with Nikon Eclipse 80i microscope.

**Results**

*S. nigrum* has been studied cytologically from 9 different localities of Parvati, Malana and Solang Valleys (Table 1). Presently 3 intraspecific cytotypes, 2x (*n*=12), 4x (*n*=24) and 6x (*n*=36) have been detected in the species. The accession scored from Solang nallha (Solang Valley) was

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Cytotype</th>
<th>Locality, province, habitat, geographical co-ordinates and altitude</th>
<th>Accession number (PUN)</th>
<th>Meiotic chromosome number 'n'</th>
<th>Meiotic behaviour</th>
<th>Pollen fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2x</td>
<td>i) Pulga</td>
<td>Parvati Valley, Kullu, Himachal Pradesh, waste places around forest borders, 31°59'38&quot;N; 77°26'28&quot;E, Alt.: 2250 m</td>
<td>56359</td>
<td>12</td>
<td>Normal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>ii) Narseng</td>
<td>Malana Valley, Kullu, Himachal Pradesh, waste places around forest borders, 32°03'55&quot;N; 77°16'11&quot;E, Alt.: 2440 m</td>
<td>56360</td>
<td>12</td>
<td>Normal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>iii) Chauki</td>
<td>Malana Valley, Kullu, Himachal Pradesh, waste places around forest borders, 32°0'31&quot;N; 77°14'43&quot;E, Alt.: 1660 m</td>
<td>56361</td>
<td>12</td>
<td>Normal</td>
<td>99</td>
</tr>
<tr>
<td>2. 4x</td>
<td>iv) Palchan</td>
<td>Solang Valley, Kullu, Himachal Pradesh, around forest area, 32°18'33&quot;N; 77°10'31&quot;E, Alt.: 2300 m</td>
<td>56390</td>
<td>24</td>
<td>Asynaptic</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>v) Dhundi</td>
<td>Solang Valley, Kullu, Himachal Pradesh, around forest area, 32°21'14&quot;N; 77°07'58&quot;E, Alt.: 2960 m</td>
<td>56391</td>
<td>24</td>
<td>Normal</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>vi) Jari</td>
<td>Parvati Valley, Kullu, Himachal Pradesh, waste places around forest borders, 32°0'10&quot;N; 77°15'17&quot;E, Alt.: 1470 m</td>
<td>56072</td>
<td>24</td>
<td>Normal</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>vii) Kasol</td>
<td>Parvati Valley, Kullu, Himachal Pradesh, waste places around forest borders, 32°0'39&quot;N; 77°19'03&quot;E, Alt.: 1570 m</td>
<td>56098</td>
<td>24</td>
<td>Normal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>viii) Tosh</td>
<td>Parvati Valley, Kullu, Himachal Pradesh, near agriculture fields, 32°0'51&quot;N; 77°27'08&quot;E, Alt.: 2430 m</td>
<td>56355</td>
<td>24</td>
<td>Normal</td>
<td>100</td>
</tr>
<tr>
<td>3. 6x</td>
<td>ix) Solang nallha</td>
<td>Solang Valley, Kullu, Himachal Pradesh, around forest area, 32°19'17&quot;N; 77°09'14&quot;E, Alt.: 2460 m</td>
<td>56392</td>
<td>36</td>
<td>Normal</td>
<td>95</td>
</tr>
</tbody>
</table>
found to be 6x, while the 2 accessions from Malana Valley and 1 accession from Parvati Valley were detected to be 2x while the rest of the 5 accessions studied from Parvati and Solang Valleys existed at 4x level. Detailed male meiotic investigations have been performed and data regarding the locality, habitat, geographical co-ordinates, altitude, accession number, meiotic chromosome number, meiotic behaviour and pollen fertility in 2x, 4x and 6x cytotypes of *S. nigrum* are provided in Table 1.

**Fig. 1.** Meiosis in *S. nigrum*; A. A PMC with 12 bivalents at M-I in 2x cytotype; B. A PMC showing 12:12 chromosomes distributions at A-I in 2x cytotype; C. A PMC showing 24 bivalents at M-I in 4x cytotype; D. A PMC showing 24:24 chromosomes distribution at two poles during M-II in 4x cytotype; E. A PMC at diakinesis showing 36 bivalents; F. A PMC at M-I showing 36 bivalents in 6x cytotype; G. A PMC showing 36:36 chromosomes distribution at 2 poles during M-II in 6x cytotype; H. A PMC at early prophase-I showing univalents; I. 48 Randomly scattered univalent chromosomes in a PMC at M-I; J. 48 Randomly scattered univalent chromosomes in a PMC at A-I; K. Univalent chromosomes did not showing congregation at the equatorial metaphase plate; L. A PMC showing eccentric position of univalents at M-I; Scale bar=10 μm.
Meiotic analysis

The 2x \((n=12)\) cytotype

The accessions from Pulga in Parvati Valley and Narseng and Chauki in Malana Valley existed at the 2x level (based on \(x=12\)) as confirmed from the presence of 12 bivalents in the PMCs at M-I (Fig.1A). These bivalents showed regular segregation of 12:12 daughter chromosomes during

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**Fig. 2.** A. A PMC at A-I showing unoriented univalent chromosomes; B. Large number of lagging chromosomes at A-I (encircled); Unequal distribution of chromosomes at A-I; C. 12:36; D. 14:34; E. 28:20; F. 25:9:14; G. 13:2:33; H. A dyad; I. A dyad with a micronucleus; J. A triad with a micronucleus; K. A polyad; L. Different sizes of unstained sterile pollen grains (micro-pollen arrowed); M. Apparently fertile normal reduced (arrowed) and unreduced (2n arrowhead) pollen grains along with an unstained sterile pollen grain; Scale bar=10 \(\mu\)m (Figs. A–K); 20 \(\mu\)m (Figs. L and M).
A-I (Fig. 1B). Further meiotic course was also regular resulting in normal tetrad formation, and high pollen fertility (99–100%).

The 4x \((n=24)\) cytotype

The 4x cytotype has been found to be predominantly more common in its distribution as confirmed from the presence of the meiotic chromosome number of \(n=24\) in 5 out of the 9 accessions scored presently from the different localities in the Kullu district. The 4x individuals in all the accessions unequivocally showed the presence of 24 bivalents in all the PMCs (Fig. 1C). Normal bivalent formation and their equal 24:24 chromosomes distribution at M-II (Fig. 1D) and anaphases resulted into high pollen fertility (98–100%).

The 6x \((n=36)\) cytotype

Only 1 accession scored from Solang nallha was found to exist at the 6x level and showed a meiotic chromosome number of \(n=36\) as confirmed from the presence of 36 bivalents during meiosis-I at diakinesis (Fig. 1E) and M-I (Fig. 1F). Equal distribution of 36:36 chromosomes at M-II (Fig. 1G) and anaphases led to normal sporad formation and high pollen fertility (95%).

Asynaptic mutant (4x cytotype)

One of the accessions scored from Solang nallha (Solang Valley) showed a complete lack of pairing between homologous chromosomes during prophase-I (Fig. 1H) and M-I (Fig. 1I). The male meiotic chromosome number in this accession was confirmed from the presence of 48 randomly distributed univalent chromosomes at M-I (Fig. 1I) and A-I (Fig.1J). Univalent chromosomes failed to congregate at the equatorial metaphase plate and remained scattered in the cytoplasm (Fig. 1K). The further course of meiosis was highly irregular, which was characterized by the eccentric position of univalents (Fig. 1L), unoriented chromosomes (Fig. 2A), lagging of large numbers of chromosomes at anaphases (Fig. 2B) and unequal distribution of chromosomes (28:20, 24 PMCs; 23:25, 9 PMCs; 13:32, 13 PMCs; 13:23, 19 PMCs; 8:40, 16 PMCs; 14:34, 21 PMCs) during anaphase-I (Fig. 2C–G). Owing to the irregular behaviour of univalent chromosomes at different stages of meiosis-I and II, microsporogenesis was also abnormal. Instead of typical sporads, irregular sporads, such as dyads, triads and tetrads (with micronuclei), and polyads were formed (Figs. 2H, I, J, K, Table 2). Lagging chromosomes constituted micronuclei during the sporad stages in all the different types of irregular sporads and produced micro/sterile pollen grains (Fig. 2L). Consequent to all these meiotic irregularities, very high pollen sterility (95%) was observed in the taxon (Fig. 2L). Also, apparently fertile and sterile pollen grains of different sizes were recorded. On the basis of a comparison of size the fertile pollen grains in abnormal 4x accession were observed to be of 2 different types (Fig. 2M, 33.04–34.75 \(\mu\text{m}\times30. 21–31.46 \mu\text{m}; 25.16–27.07 \mu\text{m}\times23.26–24.62 \mu\text{m}) compared to normal 4x plants where meiosis was perfectly normal. The large pollen grains, which measured 1.25–1.5 times bigger than the normal \(n\) reduced pollen grains, could be unreduced or \(2n\) in their constitution.

<table>
<thead>
<tr>
<th>Total no. of sporads observed</th>
<th>No. of dyads (% age)</th>
<th>No. of triads (% age)</th>
<th>No. of tetrads (% age)</th>
<th>No. of polyads (% age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>506</td>
<td>145 (28.65)</td>
<td>126 (24.90)</td>
<td>196 (38.75)</td>
<td>27 (7.70)</td>
</tr>
<tr>
<td>With micronuclei (% age)</td>
<td>49 (33.79)</td>
<td>74 (58.74)</td>
<td>14 (7.14)</td>
<td>12 (30.76)</td>
</tr>
<tr>
<td>Without micronuclei (% age)</td>
<td>96 (66.21)</td>
<td>52 (41.26)</td>
<td>182 (92.86)</td>
<td>15 (69.24)</td>
</tr>
</tbody>
</table>
Discussion

The course of meiosis in 8 of the 9 accessions among 2x, 4x, 6x cytotypes was noticed to be perfectly normal resulting into very high pollen fertility (95–100%). However, 1 accession of 4x cytotype scored from Palchan in Solang Valley depicted irregular chromosomal behaviour due to the presence of all the 48 chromosomes as univalents. The presence of high frequency of univalent chromosomes could be attributed either to the asynaptic or desynaptic nature of plants. According to Peirson et al. (1997) the majority of asynaptic mutants depict irregular distribution and random dispersion of univalents in the cytoplasm at prophase-I and M-I and they never congregate at the equatorial plate, while in the case of desynaptic mutants, bivalents and univalents orient at the equatorial plate during M-I. The present case seems to be that of a spontaneous asynaptic mutant because none of the analysed PMCs revealed the expected chromosome associations of 24II, instead exhibiting complete scattering of univalents in the PMCs at prophase-I and M-I. The univalent chromosomes were never observed to align themselves at the metaphase plate and remained dispersed in the PMCs in irregular manner. A-I/II were characterised by a highly unequal chromosome distribution at the poles. The occurrence of univalents in the asynaptic mutant during prophase I and M-I, followed by abnormalities in subsequent stages resulted in the formation of different sized microspores at the sporad stage which clearly reflected the impact of asynaptic mutation.

Meiotic irregularities resulting in the production of sterile and imbalanced gametes have been reported in several flowering plants by different researchers (Riley and Law 1965, Sjödin 1970, Gottschalk and Kaul 1980, Bione et al. 2002, Bennetzen 2002, Pandit and Babu 2003, Gaut et al. 2007) and also from this laboratory (Singhal 1982, Kumar and Singhal 2008, 2011a, 2011b, 2011c, Kumar et al. 2008a, 2008b, 2010, 2011a, b, Singhal and Kumar 2008a, 2008b, 2010, Singhal et al. 2008, 2009a, 2009b, 2010, 2011a, 2011b, Gupta et al. 2009, 2010, Himshikha et al. 2010, Kaur et al. 2010, Kumar 2011). In the present study, the high pollen sterility in one of the accessions of 4x cytotype can be ascribed to asynaptic mutation. Generally, the meiotic course in such asynaptic mutants is accompanied by a large number of meiotic anomalies such as scattered distribution of univalents in the cytoplasm, laggards at anaphases, unequal distribution of chromosomes at anaphases and multipolar PMCs. Several researchers have reported a similar type of irregular behaviour as a result of asynaptic mutations (Koduru and Rao 1981, Kaul and Murthy 1985, Singh 2002, Singhal and Kumar 2010, Kumar and Singhal 2011a, 2011b, Kumar et al. 2011a, Sharma et al. 2010, 2011).

Various factors such as drastic temperature fluctuations, soil conditions, ageing, humidity and water content, gene mutations and interspecific hybridization (Koduru and Rao 1981, Doroszewska and Berbeć 2000, Singh 2002, Rao and Kumar 2003, Trojak-Goluch and Berbeć 2003, Singhal and Kumar 2010, Kumar and Singhal 2011a, 2011b, Kumar et al. 2011a) have been reported to induce synaptic mutation in wild populations. The accession of 4x cytotype reported presently showing the asynaptic mutations had been collected from almost identical environmental conditions to those where the other 8 normal accessions of 2x, 4x, and 6x cytotypes were growing. So the role of some genetic factors in causing asynapsis could not be ruled out here. There is evidence that certain mutations change the expression of some genes controlling homologous chromosome pairing and results in the formation of synaptic mutants (Maguire and Riess 1996, Dawe 1998).

Meiotic irregularities originated as a consequence of univalent chromosomes resulting in abnormal microsporogenesis which was characterized by the presence of irregular spores with dyads, triads, tetrads (with micronuclei) and polyads. As per rule, a dyad which has 2 unreduced microspores expected to produce 2 2n (unreduced) pollen grains while a triad produces 2 2n pollen grains and 1 2n pollen. Pillay and Tenkouano (2011) were of the opinion that pollen diameter is the function of genome size since DNA content and cytoplasm increases as ploidy level increases. The status of 2n (unreduced) and n pollen was confirmed by comparing the size of pollen grains of nor-
mal 4x individuals to that of asynaptic individuals. The pollen grains having 1.25–1.5 times the linear dimensions of haploid or n pollen grains are classified here as 2n (unreduced) pollen grains as has been suggested earlier (Darlington 1937, Ortiz 1997, Oselebe et al. 2010, Xue et al. 2011).

As the 2n pollen grains are apparently fertile, there is every possibility that such 2n (unreduced) gametes can produce intraspecific polyploids as has been advocated earlier by many researchers in different plants (Falistocco et al. 1995, Ortiz 1997, Kim et al. 2009, Singhal and Kumar 2010, Kumar and Singhal 2011a, 2011b, 2012, Singhal et al. 2011a). Such 2n (unreduced) pollen grains with somatic chromosome numbers are considered to be the main driving forces for the natural polyploidization of plants. These 2n pollen grains may play an important role in the establishment of polyploid genotypes (Dewitte et al. 2010, Silva et al. 2011). S. nigrum is a highly variable species in terms of chromosome number and exist at 8 different ploidy levels (2x, 3x, 4x, 5x, 6x, 8x, 9x, 12x). Such high levels of variation in chromosome number may be attributed to frequent events of polyploid formation through sexual polyploidization. Further the authors are of the opinion that 2n pollen grains might have contributed in this direction of evolution of S. nigrum by forming the polyploid genotypes through sexual polyploidization.

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References


— and —. 2011c. Male meiosis, morphometric analysis and distribution pattern of 2n and 4x cytotypes of Ranunculus hirtellus Royle, 1834 (Ranunculaceae) from the cold regions of northwest Himalayas (India). Comp. Cytogenet. 5: 143–161.


Aust. J. Crop Sci. 4: 415–420


