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Spindle irregularities, chromatin transfer, and chromatin stickiness during male meiosis in *Anemone tetrasepala* (Ranunculaceae)

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Abstract: Detailed cytological investigations performed in *Anemone tetrasepala* Royle from the Pangi Valley revealed the presence of various irregularities (spindle irregularities, chromatin transfer, chromatin stickiness, and nonsynchronous condensation of chromatin material) for the first time in the species that existed at diploid level (2n = 14). Spindle irregularities resulted in dyads, triads, polyads, and micronuclei in sporads during microsporogenesis and variable sized pollen grains and reduced fertility in pollen grains (85.38%). Direct fusion among proximate meiocytes resulted in the formation of syncyte pollen mother cells. Such syncytes yielded jumbo-sized pollen grains that were surely of unreduced nature in their genetic constitution. Due to severe chromosome stickiness, chromosome separation during anaphases was also affected. Another interesting observation was the nonsynchronous condensation of 1–2 chromosomes during anaphase-I. This paper discusses the consequences of chromosomal and spindle irregularities on the course of meiosis and microsporogenesis and ultimately on the end products.

Key words: *Anemone tetrasepala*, nonsynchronous condensation, intra-microsporal, jumbo pollen grains, meiotic course, Pangi Valley, syncytes

1. Introduction

All living organisms, irrespective of their complex organisation, meiotically reduce their chromosome number to generate haploid gametes at the start of sexual reproduction, which compensate for fertilisation and maintain the diploid chromosome number over the generations (Golubovskaya, 1979; Pagliarini, 2000). Correct chromosome segregation is required for regular cell division and to generate balanced gametes. Meiosis, which is a crucial process for sexual reproduction in plants, is occasionally affected to a considerable extent due to certain mutations (Baker et al., 1976; Kaul & Murthy, 1985; Jiang et al., 2009). Sometimes disturbances during the meiotic course cause abnormalities in the process, which can lead to sterility of gametes as well as variation in their genetic constitution. Different types of cytological abnormalities during meiosis are known to be responsible for producing '2*n*' and variable-sized gametes with different genetic constitution have already been reported in several plants growing in the cold desert regions of Lahaul-Spiti (Kumar & Singhal, 2008, 2011a, 2011b, 2012a, 2012b; Kumar et al., 2008a, 2008b, 2010, 2011, 2012; Singhal & Kumar, 2008a, 2011b; Gupta et al., 2009), the Pangi Valley (Gupta et al., 2010; Singhal et al., 2011a, 2011b), and Kinnaur District (Singhal et al., 2008, 2011a, 2011b; Singhal & Kaur, 2009; Kaur et al., 2010). During extensive cytological surveys carried out on the plants of the Pangi Valley, we encountered the occurrence of abnormalities during male meiosis, pollen sterility, and jumbo-sized pollen grains in *Anemone tetrasepala* Royle.

A. tetrasepala Royle (family: Ranunculaceae), also treated under the genus *Anemonastrum* (*A. tetrasepalum* (Royle) Holub), is a robust perennial herb with erect hairy stems and flowers with 4–5 obovate-oblong sepals that look like petals. Basal leaves deeply 5-lobed and heart- or kidney-shaped are produced on long stalks. During May–June it bears 6–15 large sized white flowers in umbels. The species is very widely distributed and endemic to the Himalayan region, including the Kashmir Himalayas. Outside of India, the species is distributed in South and West Xizang (China), Afghanistan, and Pakistan between altitudes of 2500 m and 3400 m. Thakur et al. (2009) suggested that this species can be effectively used in landscapes owing to its beautiful white showy flowers and propagation through bulbs.

Except for a few attempts to study this endemic species for chromosome counts from the Himalayan region (Jee & Kachroo, 1985; Jee et al., 1989), no major investigation

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has been undertaken. Recently, St. Clair and Howe (2011) emphasised that endemic species should be given high priority for genetic conservation, which is not possible without proper biological knowledge. Thus it is essential to carry out detailed studies of different aspects in such endemic species. In the present case, *A. tetrasepala*, an endemic species in the Himalayas, was studied cytologically. The objectives of the present research were to study the detailed meiotic course, microsporogenesis, and effects of meiotic abnormalities encountered during different stages of meiosis-I and -II on pollen grain size and fertility in the accession collected from the Pangi Valley of Chamba District in Himachal Pradesh, India.

2. Materials and methods

Materials for male meiotic studies were collected from the wild accession during June and July 2010 from the cold desert region of the Pangi Valley (Sahali Dhar, 3300 m) in Chamba District, Himachal Pradesh, India (latitude 32°98′N; longitude 76°52′E). Voucher specimens of the cytologically studied individuals were deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN). The floral buds of suitable sizes were fixed in Carnoy's fixative (6 ethanol:3 chloroform:1 glacial acetic acid v/v/v) for 24 h and preserved in 70% ethanol in a refrigerator. Anthers from the developing buds were squashed in 1% acetocarmine and preparations were studied for detailed meiotic behaviour in pollen mother cells (PMCs) at different stages. In the accession, a total of 2400 PMCs were examined for cytological analysis and chromosome counts. Pollen studies were conducted by smearing the mature anthers from different flowers in a glycerol–acetocarmine mixture (1:1) and aniline blue (1%) dye. Five to six hundred pollen grains were analysed for pollen fertility and pollen size. Well-filled pollen grains with cytoplasm uniformly stained were considered fertile, while shrivelled pollen grains with no or lightly stained cytoplasm were counted as sterile. Pollen grain size was measured using an ocular micrometer. Wherever necessary the best slides of chromosome counts, meiotic abnormalities, abnormal sporads, and pollen grains were photographed with a Nikon Eclipse 80*i* microscope.

3. Results

The accession studied showed the meiotic chromosome number 2n = 14, as confirmed by the presence of 7 very large sized bivalents at diakinesis (Figure 1a), metaphase-I (M-I) (Figure 1b), and 7:7 chromosome distribution at anaphase-I (A-I) (Figure 1c). In spite of the normal bivalent formation and regular segregation during A-I, PMCs in the accession depicted abnormalities at different meiotic stages, which included irregular spindle activity, the phenomenon of cytomixis involving neighbouring PMCs and among the microspores within a sporad, chromosome stickiness, and nonsynchronous condensation of chromatin material. Moreover, the accession also showed the formation of syncyte PMCs.

3.1. Irregular spindle activity

The majority of the PMCs depicted normal spindle formation, which resulted in regular arrangement of bivalents at the spindle plate during M-I and segregation of chromosomes during A-I/telophase-I (T-I) and A-II/T-II. However, 17.33% (16.34 ± 1.09, mean ± standard deviation) of the observed PMCs showed irregular spindle activity, which resulted in PMCs showing the presence of a few out of plate bivalents at M-I (18.34%, 20.34 ± 2.71, Figure 1d) and the presence of lagging chromosomes (16.38%, 9.67 ± 0.93) at A-I/T-I and A-II/T-II (Figure 1e, 1f). Such PMCs also lack the ability of congregation of chromosomes at a single pole and remained scattered in the cytoplasm or in small groups (Figure 1g–1i). Unoriented chromosomes in these PMCs failed to reach the poles and constitute micronuclei during late telophase stages and sporad formation (Figure 1j–1l). Irregular spindles in these plants are also depicted in the meiocytes, which showed multipolar presence of chromosomes (8.02%, Figure 1k, 1l).

3.2. Cytomixis and chromatin transfer

The phenomenon of cytomixis involving chromatin transfer among proximate meiocytes was observed in only a few anthers within the floral bud and in only a few flower buds. In some cases during the earlier prophase stages of meiosis-I proximate PMCs are fused directly to form syncyte PMCs (Figure 2a, 2b). Although the frequency of syncyte PMCs was very low (0.27%, 3.71 ± 0.17), such syncyte PMCs were detectable during meiosis due to their large size (76.57 µm × 62.89 µm) compared to the typical PMCs (60.16 µm × 44.49 µm). After fusion, the syncytes behaved like a single large-sized PMC (Figure 2b). During the tetrad stage intra-microsporal chromatin transfer through one or more narrow and broad chromatin strands and fusion was observed in 6.86% (11.56 ± 1.24) of meiocytes (Figure 2c–2g). Occasionally, due to intra-microsporal chromatin transfer, the nuclei became fused with each other (Figure 2g) and formed a large-sized unit with double the chromatin material (Figure 2h). Consequently, microspores in the sporads with doubled chromatin material and syncyte PMCs yielded large-sized microspores, which developed into jumbo-sized pollen grains (Figure 2i).

3.3. Chromosome stickiness

During all the stages of prophase including diakinesis meiocytes did not reveal any chromatin stickiness. However, PMCs showed chromatin stickiness at M-I (35.29%, 29.76 ± 2.39), and the chromosomes appeared

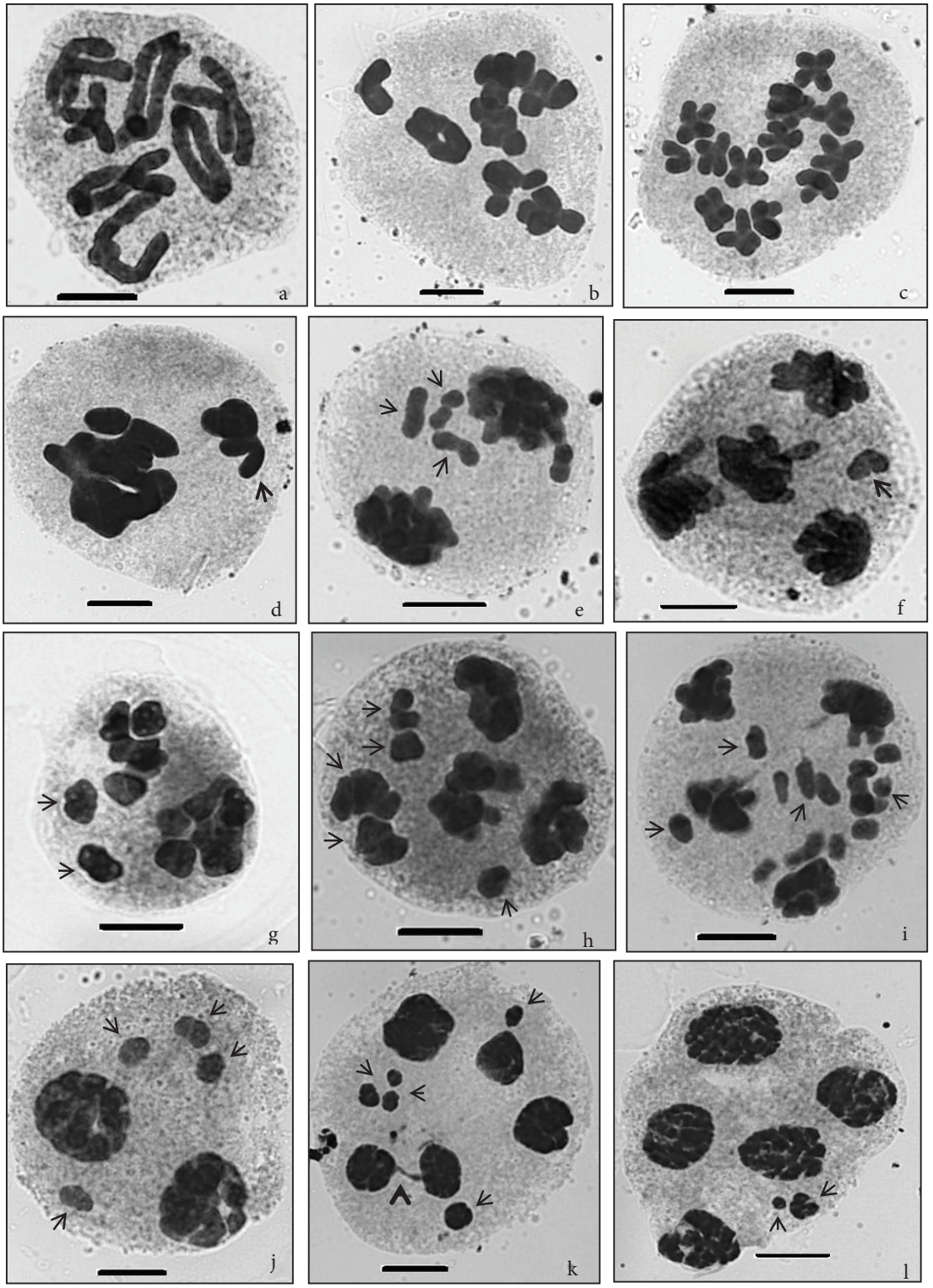

Figure 1. Meiotic chromosome number (a-c): a- diakinesis (n = 7), b- metaphase-I (n = 7), c- anaphase-I with 7:7. PMCs showing abnormal meiotic course and microsporogenesis (d-l): d- out of plate bivalents at metaphase-I (arrowed), e- lagging chromosomes at anaphase-I (arrowed), f- lagging chromosomes at anaphase-II (arrowed), g- unoriented and scattered chromosomes at anaphase-I (arrowed), h- unoriented and scattered chromosomes at anaphase-II (arrowed), i- scattered chromosomes (arrowed) in a PMC at anaphase-II, j- micronuclei at telophase-I (arrowed), k- a multipolar PMC with micronuclei (arrowed) and chromatin bridge (arrowhead) at telophase-II, l- PMC with 5 poles and micronuclei (arrowed) at telophase-II. Scale bar = 10 µm.

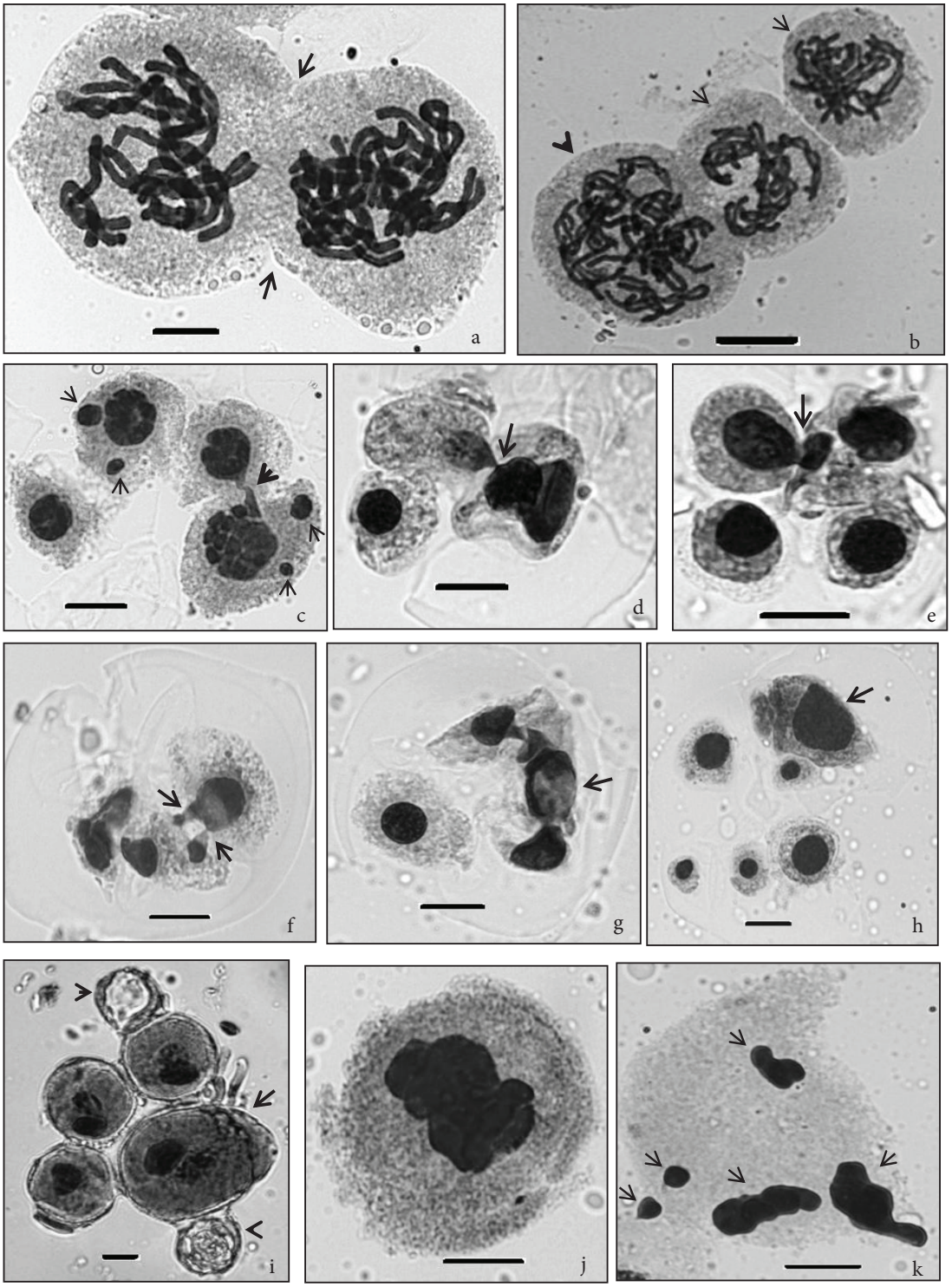

Figure 2. PMCs showing abnormal meiotic course, microsporogenesis, pollen sterility, and jumbo-sized pollen grains (a-k): a- direct fusion (arrowed) of 2 PMCs at prophase-I stage, b- a large syncyte PMC (arrowhead) and 2 typical PMCs (arrowed), c- narrow intra-microsporal chromatin material strand (arrowhead) and included micronuclei (arrowed), d- intra-microsporal chromatin material transfer by forming narrow strand (arrowed), e- transfer of chromatin material between 2 microspores through broad strand (arrowed), f- intra-microsporal chromatin material by 2 narrow strands (arrowed), g- fusion between microspores in a sporad (arrowed), h- a large microspore with almost double the chromatin material in a sporad (arrowed), i- apparently fertile stained jumbo-sized (arrowed) and typical pollen grains along with unstained sterile pollen grains (arrowhead), j- chromatin clump formed of entire chromosome complement, k- sticky chromatin in groups. Scale bar = 10 μm, except for photomicrograph b = 20 μm.

as a dense chromatin clump almost losing their identity (Figures 2j, 2k, 3a–3c). The stickiness in chromatin material persisted even during anaphases and telophases, causing difficulties in segregation of chromosomes. Due to severe chromatin stickiness chromatin bridges of different thickness were observed in the PMCs during A-I and even telophase stages (5.46%, 4.32 ± 0.96) (Figure 3a). Such chromatin bridges were also observed between the microspores at sporad stages. PMCs were observed during later stages of meiosis to undergo degeneration of chromatin material, resulting in pyknosis (Figure 3b, 3c).

3.4. Nonsynchronous condensation of chromatin material

In addition to the above-mentioned meiotic irregularities, a few PMCs in the studied plants showed unusual and nonsynchronous condensation of chromatin material in which some chromosomes showed complete condensation, while 1 or 2 chromosomes failed to be condensed. Figure 3d depicts a PMC at A-I showing 6:6 condensed chromosomes at opposite poles and 2 thread-like partially condensed chromosomes present towards the periphery of a PMC. The partially condensed chromosomes showed

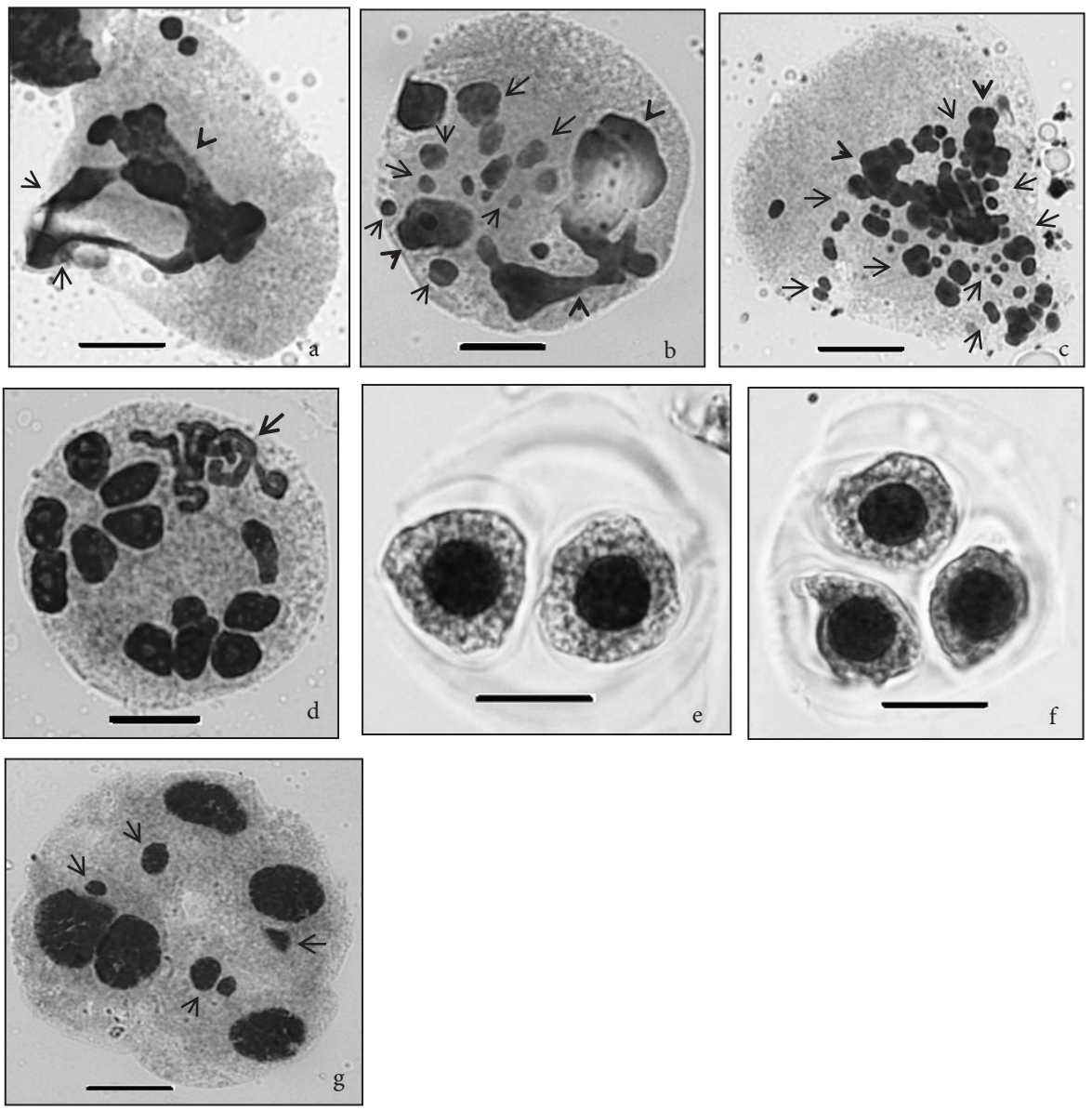

Figure 3. PMCs showing abnormal meiotic course and microsporogenesis (a-g): a- a thick sticky chromatin bridge (arrowhead) and protrusion of chromatin material (arrowed), b-chromatin stickiness (arrowhead), and degeneration and pyknosis (arrowed), c-degeneration and pyknosis of chromatin material (arrowed), d- 2 chromosomes showing nonsynchronous condensation at anaphase-I (arrowed), e- a dyad, f- a triad, g- a polyad with micronuclei (arrowed). Scale bar = 10 μm.

lagging at telophases and were not included at the poles at later stages, constituting micronuclei at the tetrad stage.

3.5. Microsporogenesis

As a consequence of anomalous chromosomal behaviour during the different stages of meiosis-I and II, microsporogenesis was also observed to be abnormal, as revealed by the presence of dyads (0.87%, Figure 3e), triads (2.74%, Figure f), polyads with micronuclei (6.83%, Figure 3g), and inclusion of micronuclei in microspores of sporads (Figure 2c). Consequently some pollen sterility (14.62% (12.83 ± 1.45)) and variable-sized pollen grains including jumbo pollen grains were recorded (Figure 2i).

3.6. Pollen grain size and fertility

On the basis of size, the pollen grains in the accession were categorised as small (16.87–22.86 µm × 16.62–22.71 µm), typical (27.01–29.94 µm × 26.80–29.73 µm), and large (31.07–37.78 µm × 30.27–35.71 µm). The large pollen grains were relatively high in frequency (69.76%) compared to the typical (26.25%) and small ones (3.51%). Besides these, a few pollen grains (0.47%) were jumbo sized (44.34–48.72 µm × 39.01–45.98 µm) (Figure 2i). Pollen grain fertility determined through stainability tests was also affected considerably and 14.62% of the pollen grains were found to be sterile with unstained/or poorly stained and shrivelled cytoplasm.

4. Discussion

The chromosome count and detailed cytological analysis were conducted in this species for the first time from the Pangi Valley; it existed at diploid level with $2n = 14$ (based on x = 7). The same diploid chromosome number of $2n = 14$ in the species was reported by Jee and Kachroo (1985) and Jee et al. (1989) from the Kashmir Himalayas in India and from outside of India by Baumberger (1971) and Ziman (2006). Furthermore, an intraspecific tetraploid cytotype with $2n = 32$ (based on x = 8) has also been reported from outside of India by Kurita (1958), which indicates the existence of intraspecific polyploidy (2x, 4x) in the species.

Aberrant meiotic course as a consequence of irregular spindle activity, chromatin transfer among neighbouring PMCs, and chromatin stickiness resulting in abnormal sporads and reduced pollen fertility has been reported in a number of flowering plants (Tilquin et al., 1984; Baum et al., 1992; Caetano-Pereira & Pagliarini, 2001; Mendes-Bonato et al., 2002; Risso-Pascotto et al., 2005; Kumar & Singhal, 2008, 2012a; Kumar et al., 2008a, 2010, 2011, 2012; Singhal & Kumar, 2008a, 2008b, 2010; Singhal et al., 2008, 2009a, 2009b, 2011a, 2011b; Singhal & Kaur, 2009; Rai & Kumar, 2010; Gulfishan et al., 2010; Himshikha et al., 2010).

Proper chromosome segregation is ensured through extensive chromosome reorganisation and the formation of a single and transient spindle during mitosis and meiosis (Caetano-Pereira & Pagliarini, 2001). Spindles that are bipolar in nature differ in their structure in different organisms; however, their basic function is to attach at specific sites called kinetochore and separate the chromosomes/chromatids at anaphases (Wadsworth et al., 2011). Before the chromosomes move to and line up at the equatorial plate it is necessary that spindle fibres attach to centromeres (Qu & Vorsa, 1999), which indicates that the function of spindle fibres is to arrange chromosomes on the equatorial plate and gather them in one group at M-I (Qu & Vorsa, 1999). Generally, irregular spindles are divided into 4 types: multipolar, monopolar, radial, and apolar (Shamina et al., 2003). According to the findings reported by Shamina et al. (2000a, 2003) multipolar spindles have 3 or more poles, and so chromosomes are randomly distributed at metaphase and then distributed into 3 or more directions at anaphase and, in the case of monopolar spindles, there exists only 1 spindle. In radial spindles ends are located at the cell periphery and near the equator (Shamina et al., 2000a), while apolar spindles have only a set of randomly oriented fibres (Shamina et al., 2000b; Seriukova et al., 2003). Formation of a bipolar spindle is essential for viable gamete production and their balanced genetic constitution. Irregular spindle activity may result in random unorientation of chromosomes in the PMCs and consequent sub-grouping of the chromosomes that function independently. A number of mutants (dv, ms28, ms43, and ms17) are reported to cause failure of spindle activity, which affects chromosome segregation (Golubovskaya & Distanova, 1986) or orientation of 2 spindles relative to each other (Golubovskaya & Sitnikova, 1980) or functional and structural disturbances of the spindle apparatus (Staiger & Cande, 1990). Recent research on *Arabidopsis thaliana* has shown that specific proteins (Multipolar Spindle 1) are involved in spindle organisation in meiocytes (Jiang et al., 2009). d'Erfurth et al. (2008) have isolated and characterised the AtPS1 (*Arabidopsis thaliana* Parallel Spindle 1) gene, which is involved in controlling the diploid ($2n$) gamete formation in *Arabidopsis thaliana* due to irregular spindle activity at male meiosis-II. In the present study, in spite of the presence of normal spindle activity in the majority of the PMCs, in 17.33% of the cases irregular spindle formation was noted. Presence of multipolar meiocytes, micronuclei in sporads, and reduced pollen fertility are the general consequence of this irregularity. In the individual studied here, irregular spindle activity also resulted in dyads, triads, polyads, and micronuclei in sporads during microsporogenesis and variable-sized pollen grains and reduced fertility in pollen grains.

The phenomenon of cytomixis, which involved the direct fusion among proximate meiocytes, resulted in the formation of syncyte PMCs. The syncyte PMCs were distinguishable because of their giant size compared to the typical ones. Further, these syncytes progressed normally through the meiotic course and yielded jumbo-sized pollen grains that were surely of unreduced nature in their genetic constitution. Similar observations regarding the formation of syncyte PMCs following the fusion of 2 or more PMCs (or nuclei) during the early prophase stages of the first meiotic division were made by Kim et al. (2009) and Singhal et al. (2011b). Interestingly, the chromatin transfer had also been observed to occur among microspores of the sporads. During this process 2 or 3 units either fuse directly or the chromatin material is first transferred from 1 microspore unit to another unit of a sporad and then fusion occurs between these units to give rise to 1 giant unit, which ultimately gives rise to jumbo-sized pollen grains. Similar intra-microsporal chromatin transfer within a sporad has also been recorded in *Clematis flammula* (Kumar et al., 2008b), *C. orientalis* (Kumar et al., 2010), *Ranunculus hirtellus* (Kumar & Singhal, 2010b), and pepper (Pozzobon et al., 2011). The exact cytological status of such jumbo-sized pollen grains produced in the presently studied species could not be ascertained herein but their '2*n*' status is clearly depicted from their size as increasing DNA content may in turn influence pollen diameter (Pundir et al., 1983; Dessauw, 1988; Jansen & Den Nijs, 1990; Tenkouano et al., 1998; Oselebe et al., 2006; Ssebuliba et al., 2008). Since these '2*n*' pollen grains are well stained/fertile, their role in the origin of intraspecific polyploids in the species could not be ruled out. It is possible that such apparently fertile '2*n*' pollen grains originating from syncytes might play a role in the origin of intraspecific polyploids in the species as has been advocated earlier in the evolution of intraspecific polyploids in *Chrysanthemum* (Kim et al., 2009), *Lindelofia longiflora* (Singhal et al., 2011b), and *Ranunculus laetus* (Kumar & Singhal, 2012a). In this paper we report for the first time the formation of syncytes and the occurrence of intra-microsporal chromatin transfer within a sporad and consequently jumbo-sized pollen grains in the species.

Chromosome stickiness was another abnormality observed during the meiotic course. Chromosome stickiness in the species involved either a few chromosomes of the complement or in some cases the entire complement. In severe cases of chromatin stickiness, the lack of chromosome separation provoked the formation of a single chromatin clump. Depending on the intensity of chromosome stickiness, pollen fertility may be partially or totally affected. Dewitte et al. (2010) in *Begonia* and Singhal and Kumar (2008a, 2010) in *Meconopsis aculeata* suggested that the chromosome stickiness has an important role in nuclear restitution as the chromatin stickiness prevents the separation of chromosomes during the anaphases and telophases. Such severe cases of chromatin stickiness resulted in restitution nuclei and yielded unreduced gametes. The phenomenon of chromatin stickiness has been reported in a number of plant species, and genetic and environmental factors and several other agents have been stated to cause chromosome stickiness. Sticky chromosomes may result due to changes in specific nonhistone proteins, as has been postulated by Gaulden (1987). In the present case, occurrence of chromosome stickiness seems to be associated with the phenomenon of cytomixis, as has been suggested in the case of *Meconopsis aculeata* (Singhal & Kumar, 2008a), *Caltha palustris* (Kumar & Singhal, 2008), and *Clematis orientalis* (Kumar et al., 2010).

In the regular course of meiosis, the chromosomes are usually condensed equally and uniformly (Bauchan et al., 1987), but in the present study 1 pair of chromosomes was found to be partially condensed at A-I, while the other 6 pairs of chromosomes were normally condensed. In the successive meiotic stages these remained as laggards and finally constituted micronuclei during the tetrad stages and yielded small sterile pollen grains. Such differential condensation of chromatin material had also been reported in *Lolium* (Jain, 1957), *Avena* (Holden & Mota, 1956), and *Agropyron cristatum* (Bauchan et al., 1987). Jain (1957) and Holden and Mota (1956) were of the opinion that nonsynchronous condensation of chromatin material is due to the lack of nucleolar activity, which might be associated with synthesis of chromosomes.

It has been demonstrated in several studies that meiosis is the most sensitive stage in the life cycle of plants and is primarily influenced by various genetic and environmental factors (Ahmad et al., 1984; Saini, 1997; Viccini & Carvalho, 2002; Sun et al., 2004; Bajpai & Singh, 2006; Rezaei et al., 2010). In the present case, the origin of various meiotic abnormalities seems to be due to low temperature stress conditions in the area where temperature falls 5–10 °C in May–June when the species enters the flowering stage. Such low temperature conditions might have affected various events of male meiosis, especially at the pre-meiotic stage, as suggested by Singhal et al. (2011b).

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