Cytomixis and Intraspecific Polyploidy (2x, 4x) in *Inula grandiflora* Willd. from Malana Valley, Kullu District, Himachal Pradesh

Himshikha, Raghbir Chand Gupta, Rohit Kumar and Vijay Kumar Singhal*

Department of Botany, Punjabi University, Patiala, Punjab 147002, India

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Summary On the basis of meiotic studies carried out presently on *Inula grandiflora* from Malana Valley in Kullu district, Himachal Pradesh, we here report the existence of cytomixis and intraspecific diploid (n=8) and tetraploid (n=16) cytotypes. The 4x individuals grow much taller and possess larger sized leaves and capitula. Stomata and pollen grain characters could also be employed in the segregation of the two cytotypes. The plants of 4x cytotype showed normal bivalent formation, regular microsporogenesis and high pollen fertility. Meanwhile, the diploid individuals which also grow under the same climatic conditions depicted the phenomenon of cytomixis involving chromatin transfer among two to eight meiocytes at different stages of meiosis. The PMCs involved in cytomixis showed various meiotic irregularities leading into reduction in some pollen fertility. Although the inter-cellular nuclear migration has been confined only to diploid individuals, which grow under the same environmental conditions, cytomixis seems to be a natural phenomenon controlled by some genes as suggested by earlier workers.

Key words *Inula grandiflora*, Intraspecific polyploidy (2x, 4x), Cytomixis, Meiotic irregularity, Malana Valley.

The genus *Inula* L. (Family: Asteraceae) with nearly 100 species is native to Europe, Asia and Africa. Approximately 20 species are reported from India. Cytological information gathered from chromosomal indexes reveals that so far only 50% of the species of *Inula* are known for chromosome counts. As a part of the project to explore the cytomorphological diversity in the plants of unexplored regions of the Northwest Himalayas, the present studies covering detailed male meiosis have been undertaken on *Inula grandiflora* Willd. on individual plant basis from the hills around Malana village in Kullu district, Himachal Pradesh. *I. grandiflora* grows as an annual herb with large sized solitary capitula of rich yellow colour which appear during late summer to early autumn. The species is mainly confined to open rocky slopes in the mountain scrubs of the Himalayas between 1800–3500 m from Western Asia to Pakistan and Nepal. Occasionally, the species has also been found growing near water sources and marshy places. Perusal of existing cytological literature reveals that so far only diploid chromosome number (2n=16) is known for the species from India (Shetty 1967) and outside of India (Sokolovskaya and Strelevskaya 1940, Gvinianidze and Avazneli 1982). The current paper reports for the first time the existence of both 2x and 4x individuals in the species from the Malana Valley. Out of these only diploid plants exhibit the phenomenon of cytomixis involving chromatin transfer among neighbouring meiocytes. The meiocytes involved in chromatin transfer also depict the presence of various meiotic irregularities. Besides the first report of cytomixis, we also suggest the identification of 2x and 4x individuals on the basis of some morphological characters like plant height and size of leaf and capitula. Even micro-characters like stomata and pollen grain size could be helpful in the segregation of the two cytotypes.

Materials and methods

Materials for male meiotic studies were collected from the wild plants growing on hill slopes around Malana Valley, Kullu district, Himachal Pradesh. Floral heads/capitula of appropriate sizes were fixed in Carnoy’s fixative (6 ethanol : 3 chloroform : 1 acetic acid, v/v/v). After 24h, the materials were transferred in 95% ethanol and stored in a refrigerator. Meiocytes preparations were made by squashing the developing anthers from florets by using the standard acetocarmine technique. For chromosome counts, PMCs (pollen mother cells) were observed under a light microscope at different stages of meiosis: late prophase-I, M-I, A-I, M-II and A-II.

For sporad analysis, anthers from florets were squashed in 1% acetocarmine. Pollen fertility was estimated through stainability tests for which anthers from mature florets were squashed in glycerol–acetocarmine.

* Corresponding author, e-mail: vksinghal53@gmail.com

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(1 : 1) mixture and 1% aniline blue dye. Well-filled pollen grains with stained nuclei and cytoplasm were scored as fertile, while shriveled ones and those with poorly stained cytoplasm were scored as sterile. Percentage pollen viability was calculated on the basis of 1000–1500 pollen grains taken from different florets/heads. Stomatal studies were made from abaxial epidermal peels obtained from the middle portion of mature leaves through KOH treatment. The epidermal peels so obtained were stained in 1% Safranin and number of stomata and epidermal cells were counted in an area of 1 mm². The stomatal index was calculated by using the following expression \[ SI = \frac{S}{(S+E)} \times 100 \] where SI stands for stomatal index, S is the number of stomata and E is the number of epidermal cells. Plants were identified by matching the accessions with the already deposited specimens lying in the herbaria of Department of Botany, Punjabi University Patiala, Forest Research Institute, Dehra Dun and Botanical Survey of India, Northern Circle, Dehra Dun. The specimens were deposited in the herbarium (Figs. 1a, 2a) maintained by Department of Botany, Punjabi University, Patiala (PUN¹). Photomicrographs of meiocytes were taken from temporary preparations using Leica Qwin imaging system and Nikon 80i Eclipse microscope.

Results and discussion

Cytological studies made on wild plants growing in Malana Valley from three localities between 1700–2700 m altitude revealed the existence of two cytotypes, the diploid \( (n=8) \) and the tetraploid \( (n=16) \). The plants of \( 2x \) and \( 4x \) cytotypes which grow under the same ecological conditions preferring open rocky slopes can be identified on the basis of plant height, size of leaf and capitula. The data on various characters (macro- and micro-) including distribution, habit and habitat and site of collection of the two cytotypes are provided in Table 1. The detailed results covering male meiotic course including microsporogenesis, pollen fertility and pollen grain size for each cytotype are given separately.

¹ PUN is the Herbarium Code of Department of Botany, Punjabi University, Patiala as per Index Herbariorum by Holmgren and Holmgren (1998).
The diploid (n=8)

The accession collected from Chaagna Village (1700m) in Malana Valley depicted a diploid chromosome count of n=8 as confirmed from the presence of eight rod-shaped bivalents at M-I (Fig. 3) and 8:8 chromosomes distribution at A-I (Fig. 4). The accession also exhibited the phenomenon of cytomixis involving chromatin transfer material among neighbouring meiocytes at early stages of prophase-I and even up to T-II (Figs. 5, 6). In some cases a chain of PMCs involved in chromatin transfer during early stages of prophase-I and even up to T-II (Figs. 5, 6). In some cases a chain of PMCs involved in chromatin transfer depicted spindle abnormalities like out of plate bivalent at M-I (Fig. 8) and non-synchronous disjunction of bivalents during A-I. Consequent to these abnormalities, the individuals showed some reduction in pollen fertility (80–89%). However, the fertile pollen grains were noticed to be of uniform size measuring 20.63×17.54 μm. Stomatal size in the cytotype is 18.85×15.67 μm (Fig. 1b).

The tetraploid (n=16)

Apparently tall-looking individuals possessing comparatively large-sized leaves and capitula were detected in two accessions gathered from hills around villages of Malana (2652m) on the way to Hydroelectric Power Project and Nerang (2700m). Such individuals depicted a tetraploid chromosome count of n=16 as confirmed from the presence of 16 bivalents at M-I (Fig. 9) and equal distribution of 16:16 chromosomes at poles during A-I (Fig. 10). In spite of the presence of early disjunction of two to three bivalents (Fig. 11), chromosome segregation and sporad formation was found to be regular leading to 100% fertile pollen grains. However, the pollen grains in 4x individuals were noticed to be of variable sizes: large-sized (26.40×25.63 μm, 60.88%) and typical sized (21.20–22.44×17.35–17.64 μm, 39.12%, Fig. 12). Stomatal size in the cytotype is 27.63×22.23 (Fig. 2b).

Chromosome counts and intraspecific polyploidy

Earlier cytological studies from India and outside of India recorded the existence of a diploid chromosome count of 2n=16 for I. grandiflora. On the basis of meiotic studies carried out on individual plant basis we have detected both the 2x and 4x individuals from Malana Valley in Kullu district, Himachal Pradesh. Occurrence of intraspecific diploid and tetraploid cytotypes has also been reported earlier in I. cappa (2n=2x, 4x), I. salicina (2n=2x, 4x), I. occulus-christi (2n=2x, 4x) and I. brittanica (2n=2x, 3x, 4x). The 2x and 4x cytotypes recorded presently could be differentiated in the field on the basis of various morphological characters (plant size, leaf size and size of capitula). The tetraploid plants grow much taller and possessed large sized leaves and capitula compared to the diploid. The 4x plants also possessed larger sized stomata and pollen grains. It is thus apparent that increase in plant height and size of leaves and capitula including micro-characters in 4x individuals is directly correlated with the increase in ploidy level as has been reported earlier in Terminalia chebula (Gill et al. 1982), Syzigium cumini (Gill et al. 1991), Centaurea phrygia (Koutekcy 2007), Centaurea stoebe (Spaniel et al. 2008, Mraz et al. 2011), Galinsoga parviflora (Bala et al. 2011), Parasenecio auriculata (Nakagawa 2006), Ranunculus hirtellus (Kumar and Singhal 2011), Spargularia diandra (Kaur and Singhal 2012), Agrimonia eupatoria (Kumar et al. 2011, Kumar et al. 2014a), Silene vulgaris (Kumar et al. 2014b) and Tordyliopsis brunonis (Kumar et al. 2014c).

Cytomixis

Individuals of both the cytotypes are found to grow...
under the same climatic conditions but only diploid plants showed the phenomenon of cytomixis involving chromatin transfer among neighbouring meiocytes. Inter-PMC transfer of chromatin material resulted into various meiotic irregularities. In the genus *Brassica*, the workers have also reported that diploid species (*Brassica compestris*) was more affected by cytomixis and cytoplasmic connections compared to polyploid species (*Brassica napus*; de Souza and Pagliarini 1997). Many other workers have also found cytomixis to be more common in diploid species (Salesses 1970, Omara 1976, Malallah and Attia 2003, Kumar et al. 2014b).

In *Withania somnifera* (Singhal and Kumar 2008b) and *Silene vulgaris* (Kumar et al. 2014b) diploid individuals showed higher frequencies of cytomixis and associated meiotic irregularities compared to the tetraploids.

Since the discovery of cytomixis, no definite explanations have been put forth regarding the consequences and possible role of cytoplasmic connections and nuclear migration. According to one of the hypotheses, the cytoplasmic channels among developing meiocytes facilitates the exchange of nutrients and informational molecules (Heslop-Harrison 1966, Mursalimov et al. 2010). Presumably, the fundamental role of cytomixis...
is the transfer of a part or whole nuclear material from donor to recipient meiocytes resulting in an increase in genetic diversity of pollen produced (Falistocco et al. 1995, Zhou 2003, Negron-Ortiz 2007). Such unbalanced and unreduced (2n) gametes could be a possible source of origin of aneuploid and polyploid plants and is of considerable evolutionary significance (Falistocco et al. 1995, Ghaffari 2006, Kumar et al. 2010, 2012, Pierre and de Sousa 2011, Mursalimov et al. 2013).

Ever since the first report of cytomixis in *Crocus sativus* (Körnicke 1901), inter-PMC transfer of chromatin has been reported earlier in a number of flowering plants, both dicots and monocots. There are conflicting views and explanations put forth by different workers. Some of the possible causes suggested include the effect of fixation (Heslop-Harrison 1966, Haroun 1995), pathological changes (Bobak and Herich 1978, Morisset 1978), pollution (Haroun et al. 2004), temperature (Narain 1976), chemical and herbicides (Ajay and Sarbhouy 1987, Bobak and Herich 1978, Haroun 1995, Kumar and Srivastava 2009), stress factors and genetic control (Ghanima and Talaat 2003). Pressure difference (Tarkowska 1965, Morisset 1978) and clumped chromatin bridges during pre-meiotic anaphases (Mendes and Rijo 1951) are the other explanations given by some workers. Considering that the plants with and without cytomixis were growing under the same climatic conditions, involvement of any environmental factor in causing cytomixis is ruled out here. In the present case, the cytomixis seems to be a natural process of inter–cellular interaction under the control of some genetic factors as mentioned by others (De and Sharma 1983, Singhal and Gill 1985, Kundu and Sharma 1988, Ghanima and Talaat 2003, Lattoo et al. 2006, Haroun 1995, Malallah and Attia 2003, Haroun et al. 2004, Kumar and Singhal 2008, Singhal and Kumar 2008a, 2010, Singhal et al. 2007, 2011, Kaur and Singhal 2012, Kaur et al. 2013, Kravets 2013, Kumar et al. 2012, 2014b, 2014c, Kumar et al. 2015).

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