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Colchicine induced manifestation of abnormal male meiosis and 2n pollen in *Trachyspermum ammi* (L.) Sprague (Apiaceae)

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Abstract. Unreduced gametes are the key source for the natural polyploidization in plants, but rate of its formation is very low in nature. Meiotic mutants are second source for the formation of 2n pollen. In this cytological investigation, the meiotic aberrations and its impact on post-meiotic products were analysed in autotetraploid *Trachyspermum ammi* (L.) Sprague (4n=36). The seedlings of *T. ammi* (L.) Sprague were treated with 3 different concentrations of colchicine (0.2, 0.4 and 0.5%, w/v) for 3 different durations. Six polyploid plants were induced which was confirmed on the basis of cytological analysis. Colchicine, an anti-microtubular drug induced different meiotic and post-meiotic abnormalities such as chromosomal bridges, lagging chromosomes, scattering, precocious, fragments, dyads, triads, and polyads. The formation of several abnormal sporads clearly signifies the meiotic restitution. The tendency of univalents to scattered in the cytoplasm at metaphase was identified as a peculiar aberration asynapsis. Pollen variability and fusion of pollen walls was reported and pollen fertility was calculated. The morphological analysis of the pollen allowed us to confirm the occurrence of 2n pollen.

Keywords: unreduced gametes, 2n pollen, polyploidy, colchicine, meiotic aberrations, *Trachyspermum ammi* (L.) Sprague.

INTRODUCTION

Ploidy induction is an effective method to induce novel quantitative and qualitative traits in plants which make them superior to their diploid ancestors. It is estimated that up to 70% of angiosperm species are polyploid, and this number is even higher if ancient polyploidization events are taken into account (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Various polyploid plants have been reported in natural ecosystems; among them some are important crop species such as potato, coffee, banana, peanut, tobacco, wheat, oats, sugarcane, and many fruits (Bretagnolle and Thompson 1995; Stebbins 1950; Udall and Wendel 2006).

Meiosis is a well-coordinated event which is vital for accomplishment of microsporogenesis. The product of male meiosis in plants is a tetrad of four

haploid microspores that are temporarily joined by a callosic wall (Brownfield and Kohler 2011). After release from the tetrad each microspore undergoes two mitotic divisions to produce a pollen grain containing the two sperm cells required for double fertilization (McCormick 2004). Whereas any disturbance to the meiotic event often has severe effects and leads to the abortion of the meiocytes or the developing gametophytes and thus sterility, a number of meiotic mutants that produce viable, unreduced gametes have been described in a range of plants (Bretagnolle and Thompson 1995; Ramanna and Jacobsen 2003).

Gametes with somatic chromosome numbers referred to as unreduced ($2n$) gametes. According to Bretagnolle and Thompson (1995), the production of unreduced gametes is considered the main cause of polyploid induction in nature. The unreduced ($2n$) gametes can be formed due to both pre- and post-meiotic genome duplication events, as well as meiotic restitution (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). The heterozygosity within $2n$ gametes depends on its cytological mechanism which is subdivided as first division restitution (FDR), second division restitution (SDR) and indeterminate meiotic restitution (IMR) (Ramanna and Jacobsen, 2003). An easier diagrammatic representation of restitution mechanism was given by Ferris *et al.* (1992). A FDR $2n$ gamete comprises non-sister chromatids, whereas a SDR $2n$ gamete comprises two sister chromatids (Tang 2002). The third (IMR) type was observed in lily, carried characteristics similar to both SDR and FDR (Lim *et al.* 2001). In this intermediate type, both univalents and bivalents are formed during metaphase I.

Crossing $2n$ pollen with a normal female gamete has been shown to be one of the most effective methods to produce triploid individuals (Nilsson-Ehle 1936; Müntzing 1936). According to Dewitte *et al.* (2012), meiotic mutants are a second source of $2n$ gametes. Yet, it has been considered that the rate of occurrence of $2n$ pollen in nature is very low hence the production rate of polyploids was less than 0.1% (Liu *et al.* 2019). To increase the occurrence rate of $2n$ pollen, novel methods for inducing polyploid production in plants *via* the use of spindle inhibitors, such as dinitroanilines or colchicine, have been successfully explored and used in some species (Vaughn and Lehnen 1991; Hancock 1997; Zlesak *et al.* 2005; Dhooghe *et al.* 2009). Amongst several antimicrotubular drugs, colchicine is traditionally and preferably being used as doubling agent (Kumar and Dwivedi 2017). A widespread data has been accumulated over the last 60 years which specifies that the drug colchicine manifest defects in meiotic prophase. It reduces the fre-

quency of chiasmata (Driscoll and Darvey 1970, Shepard *et al.* 1974) and impairs synaptonemal complex formation (Tepperberg *et al.* 1997).

The large number of world's population rely either solely or largely on traditional herbal remedies for health care (Dwivedi 2016). One of these important medicinal plants is ajwain (*Trachyspermum ammi* (L.) Sprague, $2n = 2x = 18$) which is a rich source of various neutral components, due to which it occupies a significant economic position in pharmaceutical industries (Dwivedi and Kumar 2018). Many of the medicinal and aromatic plants do not have stable production in their growing areas and are usually gathered in accordance with conventional methods to meet demands (Dalkani *et al.* 2012). Therefore to meet the growing pharmaceutical demands, attention need to be paid to medicinal plants with stable quality. $2n$ gametes are an effective and efficient way to transmit genetic diversity to the plants, including both valuable qualitative and quantitative traits (Peloquin *et al.* 1999). Recently, plant breeders have become interested in the practical use of $2n$ gametes in breeding program due to the new tools available for $2n$ gamete manipulation and insights into the genetic background of their formation (Dewitte *et al.* 2012). However artificial manipulation of ploidy in ajwain has been previously achieved by few researchers (Kumar and Dwivedi 2017 and Noori *et al.* 2017) yet the potential role of unreduced gametes to create genetic variability in this crop is unmapped. Hence, through this study we had made an effort to understand the effect of *in vitro* colchicine-induced disruption in meiotic products, mechanism of formation of $2n$ pollen and its repercussions on the crop fertility.

MATERIAL AND METHODS

Plant material

Fresh and healthy seeds of *T. ammi* var. AA-1 were procured from National Research Centre for Seed Spices, Ajmer, Rajasthan, India. Seeds were sown in earthen pots in triplicates during October to November season to raise the seedlings.

Agro-climatic conditions of experimental site

The present experiment has been performed in the area of Roxburgh Botanical Garden, Department of Botany, University of Allahabad, Prayagraj, U.P., India, during the rabi season. The exact experimental location is 25°27'43.01"N, 81°51'10.42"E. Prayagraj is situated 98 m

above mean sea level. Prayagraj is in the sub-tropical climatic zone; the average rainfall is 1027 mm and relative humidity is 59%.

Induction of autotetraploidy

For the present work colchicine ($C_{22}H_{25}NPO_6$) manufactured by Himedia Laboratory Pvt. Ltd., Mumbai, India was used. The treatment was given to the seedlings within 2–3 days of germination (before third leaf emergence). Emerging shoot apices at cotyledonary stage of each of the 20 seedlings/pot were treated with aqueous solution of 0.2, 0.4, and 0.6% concentration by cotton plug method for one, two and three consecutive days with an alternate recovery time period of 12 hours in each case. A set of control was also maintained of 20 plants/ pot.

Meiotic preparation

Small sized floral buds were fixed in Carnoy's fixative viz. glacial acetic acid: ethyl alcohol (1 : 3, v/v) and then transferred to 70% alcohol after 24 hours and stored at 4°C until use. Meiotic slides were prepared by using anther squash technique with 2% standard acetocarmine stain, observed and microphotographed under Nikon Phase Contrast Research microscope (Nikon Eclipse, E200, Japan) at 40X magnification.

Pollen viability assessment

Pollen viability was estimated using a glyceracetocarmine i.e. stained cytoplasm with nucleus were considered as fertile whereas unstained and shriveled pollen grains without nuclei were considered as sterile.

Statistical analysis

All the statistical analyses of morphological and cytological observations have been done by using SPSS 16.0 software to measure mean values of variables. A pair wise comparison of means was made using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ significance level.

RESULTS

The diploid plant of *Trachyspermum ammi* (L.) Sprague of var. AA-1, used in the present study, had

$2n=18$ ($n=9$) as confirmed in mitotic as well as meiotic studies (Figure 1.1). The meiotic behaviour of six induced polyploid plants of ajwain were analysed and found that the number of bivalents was more than 9 at early prophase (Figure 1.2). Moreover, the PMCs of colchicine treated plants showed various chromosomal aberrations such as unorientation, laggard (Figure 1.9 and 1.11), bridge (Figure 1.9), scattering (Figure 1.4 and 1.10), precocious movement (Figure 1.7 and 1.8), fragment (Figure 1.3 and 1.10), etc. The range of these aberrations was higher in metaphase I and II as compared

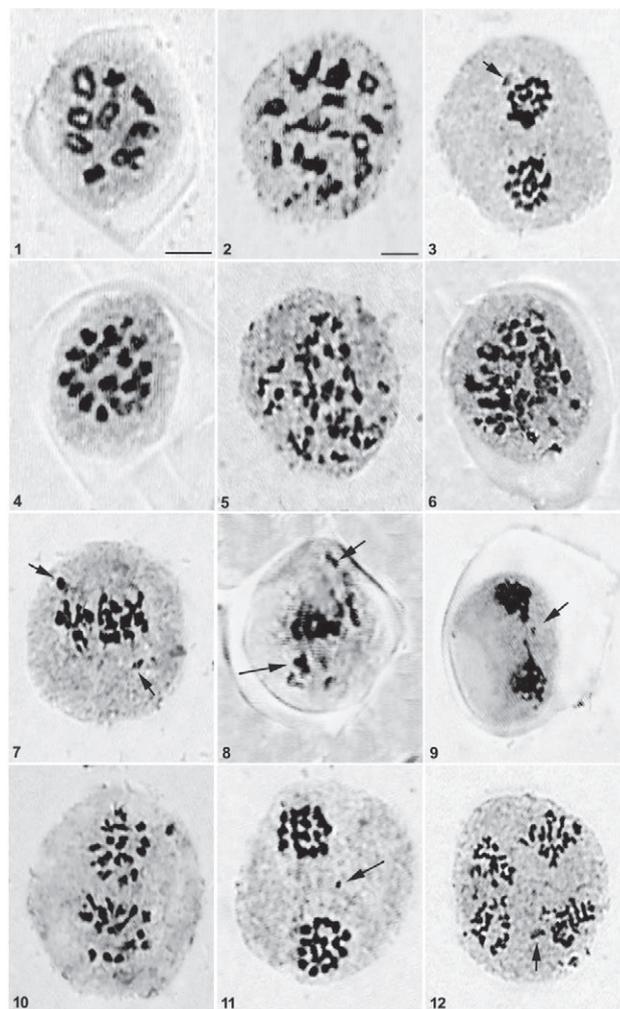


Figure 1. 1. A PMC showing 9 bivalents at diakinesis (diploid), 2-12. Meiotic stages of autotetraploid plants ($4n=36$); 2. Normal PMC at early prophase, 3. Fragment at anaphase I, 4. Scattering at metaphase I, 5 and 6. Meiotic configurations of asynaptic plants, 5. $2IV+5II+10I$, 6. $5II+26I$, 7 and 8. Precocious movement at metaphase I, 9. Bridge formation alongwith laggard at sticky anaphase I, 10. Scattering and fragment at anaphase I, 11. Laggard at anaphase I, 12. Fragment at anaphase II. Scale: $10\mu m$

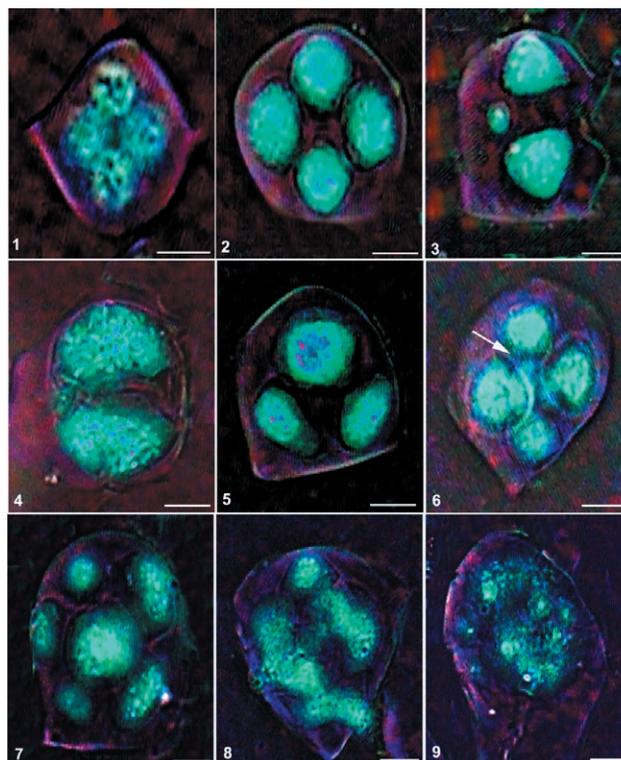
Table 1. Effect of colchicine on different meiotic phases of *Trachyspermum ammi* (L.) Sprague.

Concentration (%)	Duration (hours)	Plant No.	Meiotic Abnormalities (Mean±S.E.)		
			Meta I/II	Ana I/II	TMA
Control	-	P1-P10	-	-	-
0.2	24	P-1	4.22±0.58 ^a	3.28±0.26 ^{ab}	7.50±0.79 ^a
		P-2	5.07±0.80 ^{ab}	4.26±0.26 ^{ab}	9.33±1.68 ^{ab}
	36	P-3	5.50±0.39 ^{ab}	4.27±0.38 ^{ab}	9.77±0.69 ^{ab}
		P-4	6.68±0.34 ^b	2.97±0.29 ^a	9.65±0.41 ^{ab}
0.4	24	P-5	5.47±0.17 ^{ab}	4.50±0.14 ^{bc}	9.97±0.28 ^{ab}
0.6	12	P-6	5.72±0.47 ^{ab}	4.79±0.76 ^c	10.51±1.22 ^b

*Mean±S.E., Values followed by the superscript differ at $p < 0.05$ between treatments by the DMRT.

to both divisions of anaphase. The dividing phases of plants were less affected by lower doses of colchicine (24 and 36 hours treatment of 0.2% concentration) while it was higher in case of 0.4% and 0.6% concentrations. The highest total meiotic aberration (TMA) was observed at 0.6% concentration (10.51±1.22%) however it was range from 7.50±0.79% to 9.77±0.69% at 0.2% concentration (Table 1). The tendency of univalents to scattered in the cytoplasm at metaphase exhibited the peculiar phenomenon of asynapsis which was witnessed at 0.4% concentration of colchicine (figure 1.5 and 1.6).

Owing to the outcomes of these meiotic aberrations, abnormal post-meiotic products have been reported among which triads showed predominance over the dyads and polyads. An increasing trend for polyads formation was recorded with respect to colchicine *i.e.* 4.19±0.42% to 8.20±0.51% in a dose dependent manner (Table 2). The arrangement of these sporads was quite different from the normal tetrad. Figure 2.3 showed dyad with one small microcyte unlike the

**Figure 2.** 1. Normal tetrad of diploid plant, 2. Normal tetrad of autotetraploid plant, 3. Dyad with a microcyte, 4. Dyad, 5. Triad, 6. Pentad, 7. Polyads, 8. A polyad with fused microspore in two groups, 9. a polyad completely fused microspore. Scale: 10µm

dyad shown in figure 2.4. In some instances, the polyads were recorded in which the sporads were not joined by callosic wall (figure 2.8 and 2.9) unlike the normal tetrad stage. Such sporads can produce gametes of different ploidy levels. As a consequence of these abnormal sporads, meiotic index (MI) was decreased along with increasing the concentrations of colchicine. The MI was

Table 2. Effect of colchicine on post-meiotic products, meiotic index and Pollen fertility of *Trachyspermum ammi* (L.) Sprague.

Concentration (%)	Duration (hours)	Plant No.	Post-meiotic Abnormalities (Mean±S.E.)			MI (%)	PF (%)
			Dyads	Triads	Polyads		
Control	-	P1-P10	-	1.75±0.24 ^a	-	98.72±0.33 ^f	98.99±0.22 ^f
0.2	24	P-1	7.80±0.43 ^b	21.35±0.09 ^b	4.19±0.42 ^b	64.86±0.30 ^e	59.30±0.20 ^e
		P-2	8.16±0.36 ^b	21.49±0.33 ^b	5.69±0.43 ^c	62.11±0.15 ^c	58.80±0.17 ^d
	36	P-3	8.20±0.64 ^b	21.92±0.42 ^{bc}	6.43±0.47 ^c	64.05±0.07 ^d	58.32±0.22 ^{cd}
		P-4	8.70±0.42 ^{bc}	22.96±0.47 ^{cd}	6.15±0.41 ^c	63.40±0.22 ^d	58.29±0.25 ^c
0.4	24	P-5	8.80±0.40 ^{bc}	23.64±0.26 ^d	6.52±0.60 ^c	57.20±0.19 ^b	41.20±0.14 ^b
0.6	12	P-6	9.75±0.43 ^c	24.03±0.52 ^d	8.20±0.51 ^d	55.10±0.20 ^a	39.10±0.09 ^a

TMA- Total Meiotic Abnormalities, MI-Meiotic Index, PF- Pollen Fertility.

*Mean±S.E., Values followed by the superscript differ at $p < 0.05$ between treatments by the DMRT.



Figure 3. 1. A diploid pollen ($2n$), 2-4. Different autotetraploid pollen grains ($4n$), 2. a fertile tetraploid pollen 3. a sterile budding pollen grain, 4. A fertile pollen showing variability in shape. Scale: $10\mu\text{m}$.

recorded as $64.86\pm 0.30\%$ at 24 hour duration of 0.2% concentration whereas $55.10\pm 0.20\%$ at 0.6% concentration of colchicine (Table 2).

On account of these aberrant post meiotic products, the process of microsporogenesis is significantly affected and consequently resulted variability in pollen grains (Figure 3). The pollen grains exhibited variability in terms of their shape and size. The shape was observed to be remarkably transformed which is represented in figure 3. The fertility of pollen grains were gradually reduced (Table 2) and ranged from $59.30\pm 0.20\%$ (at 0.2% concentration) to $39.10\pm 0.09\%$ (at 0.6% concentration). Figure 4.1 and 4.2 represents the pollen cytomixis in which the chromatin material was transferred through both wide and narrow channels to the proximate pollens. Onset of passing event was evident by the assemblies of pollens where the dissolution of walls was observed (Figure 4.2). Direct fusion of pollen grains is represented in figure 4.1. The size of pollen grain of diploids was registered as $4.42 \pm 0.04 \mu\text{m} \times 2.67 \pm 0.12 \mu\text{m}$ while $7.09\pm 0.17 \mu\text{m} \times 5.31\pm 0.20 \mu\text{m}$ in case of autotetraploids. The pollens of polyploids were near about two times larger as compared

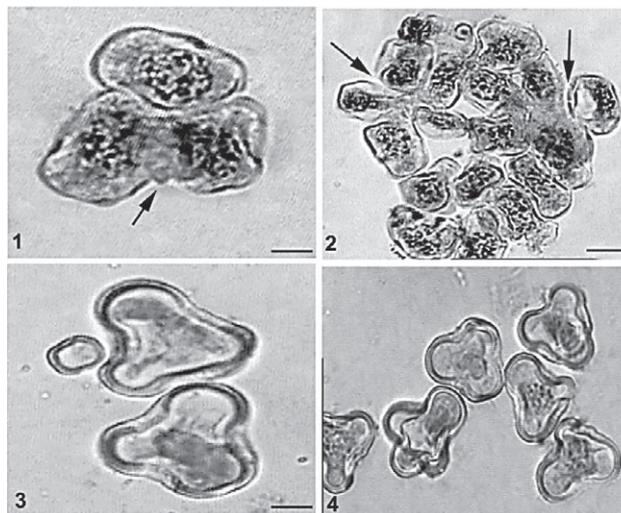


Figure 4. 1. Fusion of wall between two pollen grains, 2. Fusion of walls in a group of pollen grains, 3 and 4. Pollen variability. Scale: $10\mu\text{m}$.

to diploids thus it was considered as unreduced pollen ($2n$) (figure 3.2 and and 4.1-4.4).

DISCUSSION

The repercussions of colchicine induced meiotic aberrations resulted to genetically unstable polyploid plants. The proper spindle formation and its precise ongoing activity during whole meiotic event are essential for the accomplishment of fertile progeny. However, Levan (1939) stated that colchicine causes a temporary inactivation of the spindle mechanism without damaging any other life processes of the chromosomes. Thus, testing the stability of induced polyploids is critical and should be studied at various stages as the plant material matures (Harbard *et al.* 2012).

Meiotic prophase I is characterized by chromosome cohesion, pairing, and recombination (Ma, 2006). These bivalents are the physical sites of crossover between homologous chromosomes and are only established if pairing and recombination occur normally. The mutations concerning to meiotic prophase I that often result in univalents (paired sister chromatids) rather than bivalents (Ross *et al.* 1997; Bai *et al.* 1999; Bhatt *et al.* 1999; Couteau *et al.* 1999; Caryl *et al.* 2000; Grelon *et al.* 2001; De Muyt *et al.* 2009). The unequal segregation of univalents in meiosis I followed by an equal second division, results in the formation of aneuploid cells which abort during development, however in some mutants, a few functional gametes are produced (Cou-

teau *et al.* 1999; Azumi *et al.* 2002; Ravi *et al.* 2008). In both meiosis I and II, bridge formation accompanied with the fragment/s was exhibited at the higher concentration of colchicine which attributed to the presence of paracentric inversion (Sybenga, 1996). Chromosome bridges have often been studied by observing cancer cells containing chromosomes damaged by spontaneous telomere loss (Fouladi *et al.* 2000; Lo *et al.* 2002; Acilan *et al.* 2007). These damaged chromosomes enter the breakage–fusion–bridge cycle (Zheng *et al.* 1999). Bajer (1964) mentioned that chromatin bridges are responsible for retardation of kinetochore movement, or, frequent bridges formation may result in nuclear restitution. In the present study, the phenomenon of asynapsis has been observed which showed the inability of univalents to assemble at the equatorial plate and widely scattered in cytoplasm at metaphase stage suggests lack of pairing at early prophase. According to Gottschalk and Kaul (1980 a, b), the absence or failure of synapsis is termed as asynapsis. The asynaptic mutant induced by colchicine was previously described in soybean by Kumar and Rai (2007); proposed that this mutation might have affected specific genes for chromosome pairing.

The sporads are highly specialized cells which are able to produce four haploid cells after a series of genetically controlled steps (Caetano-Pereira *et al.* 1999). The elimination or addition of one or more chromosomes (as a consequence of laggard, precocious, bridge, fragments, *etc.*) is responsible for the formation of anomalous post-meiotic products such as monads, dyads, triads and polyads resulted to the unreduced gametes (Golubovskaya 1989) or sterile (Bosco *et al.* 1999) pollen grains. According to Kiihl *et al.* (2011), the meiotic phases that precede cytoplasm cleavage might have caused failure in the cytokinesis process resulting into monads, dyads, triads and polyads. There are three main types of abnormal spindles orientation *i.e.* parallel, fused and tripolar that have been reported to produce 2n pollen (Mok and Peloquin 1975; Veilleux 1985; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Zhang *et al.* 2009; Zhang and Kang 2010; Silva *et al.* 2011). The parallel spindles resulted in two FDR 2n pollens while fused spindles are accountable to form a dyad and then two FDR 2n pollens. The tripolar spindles develop a mother cell to produce one FDR 2n pollen and two 1n pollen (Zhang and Kang 2013).

However, Ramanna and Jacobson (2003) stated that FDR is typical in synaptic mutants or distant hybrids, in which homologous chromosome pairing (bivalent formation) is completely absent, although other mechanisms, such as cytokinesis failure or spindle abnormalities during metaphase II, can also lead to an equiva-

lent of FDR. FDR gametes contain an equal number of parental chromosomes due to the equatorial division of sister chromatids. Therefore, FDR pollen are key entities in producing heterozygous hybrids, because of the highly heterozygous 2n gametes formed (Bretagnolle and Thompson 1995). On the contrary, sister chromosomes move to the same daughter cell in case of SDR gametes, thus due to the absence of crossing-over, it exhibit maximum homozygosity (Hermsen 1984; Veilleux 1985; Peloquin *et al.* 1999). Owing to the abnormal spindle activity, the unreduced pollen of *T. ammi* genetically represents the FDR mechanism.

Naturally, the establishment of new polyploid genotypes is infeasible without the existence of 2n gametes. Thus its role has great significance in evolution, as the cytological mechanisms of 2n gamete formation demonstrate that it might be the source of variable genetic combinations. Since, our investigation was performed only to determine the cytological behavior of unreduced gametes; further researches are required to understand the extent of heterozygosity of 2n gametes and its influential role to create variability in ajwain crop.

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