Cytomixis in Pollen Mother Cells of *Medicago sativa* L.

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Abstract

Cytomixis (i.e., chromatin migration between meiocytes) has been detected in many plant species, but not in *Medicago sativa* spp. In the present study we report the identification of a few cytomictic alfalfa plants. Those plants, the “mother plants,” were selfed and crossed with a normal control plant. Microsporogenesis analysis was performed on the mother plants, on the S1 and F1 plants, and on controls. The S1 and F1 plants, like the mother plants, were found to be cytomictic. Single or multiple chromatin bridges between two or more meiocytes were observed almost exclusively in prophase I. Some completely empty meiocytes were also observed. In addition to cytomixis, other meiotic abnormalities were found. Control plants showed an almost regular meiosis. The highest values of cytomixis were observed in the mother plants, and the lowest in their F1 progenies. Variability of cytomixis in the F1 plants is probably due to a heterozygotic condition of the parents for this trait. No significant correlation was found between cytomixis and pollen viability, even if the cytomictic plants showed low values of pollen viability.

Cytomixis is defined as the migration of chromatin between adjacent cells through cytoplasmic connection channels and was first recorded by Körmicke (1901) in pollen mother cells (PMCs) of *Crocus sativus*. It occurs in a great number of plant species (Cheng et al. 1975; Gottschalk 1970; Omara 1976) and has mainly been observed in PMCs, but also in the tapetal cells (Cooper 1952) and in the ovary cells of various plants (Koul 1990). Cytoplasmic connections between meiocytes originate from the preexisting system of plasmodesmata which develops in anther tissues and then, in general, becomes obstructed by the progressive deposition of callose (Heslop-Harrison 1966). However, the plasmodesmata may sometimes persist during meiosis and so increase in size as to generate intermeiotic connections known as cytomiotic channels. Cytoplasmic organelles and/or chromatin may pass through those channels (Risueño et al. 1969). Cytomixis has been more frequently observed during the microsporogenesis of genetically unbalanced plants such as haploids, aneuploids, hybrids (de Nettancourt and Grant 1964), mutants (Gottschalk 1970), triploids (Salesses 1970), and apomicts (Mantu and Sharma 1983). In some species, polyploid forms seem to exhibit more cytomixis than their diploid counterparts (Semyarkhina and Kuptsou 1974), though cytomixis has also been observed in diploid types (Sapre 1978).

Although cytomixis is a widespread phenomenon and has been extensively studied in a great number of species, its origin and significance are still unclear and its role in evolution as well as its genetic control remain speculative and controversial. While cytomixis was considered in the past to be an anomaly, either pathological (Morisset 1978) or induced by fixation or by traumatic injury (Takats 1959), it is now usually considered to be a normal, though infrequent, cytological phenomenon and not an artifact of preparations. Some of the factors thought to be responsible for cytomixis include the action of chemical agents such as colchicine (Dwivedi et al. 1988), the use of herbicides (Bobak and Herich 1978), the partial or total inhibition of cytokinesis during microsporogenesis (Risueño et al. 1969), changes in the biochemical processes that involve microsporogenesis modifying the microenvironment of affected anthers (Koul 1990), and finally, the influence of genes (Bedi 1990). According to Nirmala and Kaul (1994), cytomixis as observed in *Pisum sativum* would be caused by a male-sterile mutant gene and its frequency would be altered by environmental factors. Whether a spontaneous or an induced process, cytomixis may have serious genetic consequences, such as the formation of PMCs with anomalous chromosome numbers, or binucleated PMCs, and of aberrant microspores (triads, pentads, hexades), pollen sterility (Soodan and Waffai 1987), chromosome stickiness, and syncytia (Patra et al. 1986). All these abnormalities interfere negatively with fertility and with the reproductive potential, and might even modify the reproductive system (Soodan and Waffai 1987).
Adriana 2
3
3
Adriana 2
Adriana 2
Lodi 8
3
Lodi 6
3
Lodi 5
3
Lodi 4
6
Lodi 2
3
Crosses (mean of five plants)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Prophase I with cytomixis (%)</th>
<th>Pollen viability (%)</th>
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<tbody>
<tr>
<td>Controls</td>
<td></td>
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| Cultivar Adriana  
(mean of five plants) | 1.1 ± 0.2 a | 94.2 ± 1.8 a |
| Adriana 2 (s.p.) | 0.5 ± 0.6 | 98.3 ± 0.8 |
| Mother plants |                               |                      |
| Lodi 2 (s.p.)  | 27.7 ± 1.3 | 34.5 ± 3.1 |
| Lodi 4 (s.p.)  | 36.0 ± 1.6 | 46.9 ± 1.5 |
| Lodi 5 (s.p.)  | 49.4 ± 2.1 | 65.0 ± 2.1 |
| Lodi 6 (s.p.)  | 66.3 ± 0.8 | 61.3 ± 1.8 |
| Lodi 8 (s.p.)  | 50.0 ± 1.1 | 62.8 ± 0.7 |
| Mean          | 45.9 ± 6.6 b | 54.1 ± 5.8 b |
| Self progeny (mean of five plants) |       |            |
| Lodi 2 self   | 24.7 ± 3.2 c | 30.7 ± 4.6 c |
| Lodi 4 self   | 32.4 ± 4.1 b | 40.2 ± 5.1 b |
| Lodi 5 self   | 40.0 ± 3.8 b | 60.3 ± 3.6 b |
| Lodi 6 self   | 32.8 ± 5.5 b | 60.0 ± 4.2 b |
| Lodi 8 self   | 38.5 ± 3.2 b | 60.9 ± 3.7 b |
| Crosses (mean of five plants) |       |            |
| Lodi 2 × Adriana 2 | 15.8 ± 8.1 a | 85.6 ± 3.0 c |
| Lodi 4 × Adriana 2 | 13.2 ± 3.5 c | 90.4 ± 1.4 a |
| Lodi 5 × Adriana 2 | 38.7 ± 4.6 b | 69.6 ± 6.0 b |
| Lodi 6 × Adriana 2 | 39.8 ± 5.3 b | 81.6 ± 2.6 d |
| Lodi 8 × Adriana 2 | 13.6 ± 4.6 a | 91.8 ± 1.9 a |
| Adriana 2 × Lodi 2 | 11.4 ± 3.4 c | 77.0 ± 4.6 c |
| Adriana 2 × Lodi 4 | 18.2 ± 6.0 c | 80.8 ± 4.3 c |
| Adriana 2 × Lodi 5 | 38.8 ± 8.7 b | 66.8 ± 4.3 b |
| Adriana 2 × Lodi 6 | 25.0 ± 2.9 c | 80.2 ± 2.9 c |
| Adriana 2 × Lodi 8 | 25.4 ± 12.8 ab | 85.4 ± 6.1 a |

Plants were grown in a field trial in central Italy (Perugia)
Results are expressed as mean ± SE. For Adriana 2 and the mother plants, each value is the mean of three measurements.
s.p., single plant.
The mean values of cytomixis and pollen viability in the S1 and F1 progenies were analyzed using the t test versus either the mean value of the control plants or the mean value of the mother plants.
In each column, values followed by the letter a or b are not significantly different (P ≤ 0.05) from the values recorded for the control or the mother plants, respectively. We indicated with c all the values significantly different from those indicated with a or b, but we did not analyze the difference between means indicated with c.

While studying an alfalfa (Medicago sativa L., 2n = 4x = 32) population, we observed cytomixis for the first time in that species in some plants characterized by very little or no seed production. Therefore the purpose of the present investigation was to analyze the meiotic behavior of those plants.

Materials and Methods
Tetraploid plants with very low seed production were identified in a population of M. sativa, cv. Adriana. All of these plants, designated L2, L4, L5, L6, L8 (the mother plants), were found to be cytomictic. Five more tetraploid plants from the same population as the mother plants, with normal seed production and regular meiosis, were also analyzed as controls. In order to study the inheritance of cytomixis, the cytomictic plants were selfed and crossed as both male and female parents with one of the five control plants (Adriana 2) under controlled conditions. Cytological analyses were performed on the mother plants, on the S1 and F1 plants, and on the control plants.

In order to check for possible environmental effects on cytomixis, the mother plants and the control plants were both grown in two different areas, at Perugia (central Italy) and at Lodi (northern Italy), and no treatments with either pesticides or herbicides were used. Moreover, for analysis of meiosis, the inflorescences were collected at two different times of the year, at the end of May and at the beginning of September, in order to verify the persistence of possible meiotic abnormalities. Data in Table 1 indicate the values calculated for the samples collected in May. The values obtained for the inflorescences collected in September were very similar to the May samples and therefore are not shown. Young inflorescences were fixed in ethanol-acetic acid (3:1) at 4°C and then stained according to the Feulgen method (hydrolysis in HCl 1 N for 8 min and basic fuchsin for 10–15 min). The anthers of each flower were squashed in a drop of 2% acetic orcein to intensify the staining of meiotic cells. Cytological analysis of the meiotic phases was performed on a sample of 500 cells per plant. Pollen was collected from the same plants as those used for meiotic analysis. Pollen viability was determined on 1600 pollen grains per plant on slides prepared with 2% acetic carmine and glycerin (1:1).

The data were submitted to analysis of variance (ANOVA) and the differences between two means were tested using the t test of significance according to Snedecor and Cochran (1967). Each mean value for cytomixis and pollen viability of the S1 and F1 progenies was analyzed using the t test versus either the mean value of the control plants or the mean value of the mother plants (Table 1). We did not analyze the differences between means within the groups of S1 and F1 plants.

Results and Discussion
Microsporogenesis analysis revealed cytomixis, that is, the migration of chromatin between meiocytes through cytoplasmic channels, both in the mother plants and in their S1 and F1 progenies. Chromatin migration caused the formation of single or multiple chromatin bridges (Figure 1a,b), which involved two or more cells (Figure 1c,d). In some cases the intercellular connections were so numerous as to induce an aggregation of the meiocytes, which most probably were unable to complete meiosis. Moreover, numerous PMCs were observed, which had chromatin masses against the cell membrane or in loose order in the cell (Figure 1e). We occasionally observed meiocytes that were completely empty following total chromatin migration into another meiocyte or,
more rarely, outside the cell (Figure 1f). It is very probable that the meiocytes with no chromatin are lost during the meiotic divisions, whereas those with an unusual chromatin content determine abnormal microspore development and the formation of more- or less-viable gametes with an unbalanced chromosome number. The formation of 2n gametes as a consequence of chromatin migration between meiocytes cannot be excluded either (Falistocco et al. 1995). Thus cytomixis could be considered a possible source of polyploid or aneuploid plants (Srivastav and Raina 1980).

Figure 1. Cytomixis in PMCs of *M. sativa* plants. Meiocytes in prophase I with (a) single or (b) multiple chromatin bridges. Chromatin migration between (c) two or (d) more cells in prophase I. (e) PMCs with chromatin masses distributed throughout the cytoplasm. (f) Total chromatin migration from one meiocyte to another. Chromatin bridges between cells in (g) first and (h) second meiotic divisions. Meiotic abnormalities, other than cytomixis, in first (i) meiotic divisions of cytomictic plants. (j) Normal first and second meiotic divisions in the control plants. Low and high pollen viability in the (k) cytomictic and (l) control plants, respectively. (Magnification: a–h, 500×; i,j, 320×; k,l, 200×.)
In our plants, chromatin transfer between PMCs was seen almost exclusively at the prophase of first meiotic division (Figure 1g), while it was very rare in the subsequent phases of first division and in the second meiotic division (Figure 1h). This finding agrees with results obtained in other species (Guo et al. 1987; Yang et al. 1993) and leads us to assume that cytomixis could be originated by an anaphase delay during the mitosis preceding meiosis, which would cause an aberrant meiotic chromosome segregation, and that the genes involved in cytomixis regulation are the same genes that are responsible for meiotic chromosome segregation, like the DIF-1 in Arabidopsis thaliana for example (Bhatt et al. 1999). However, cytomixis has also been observed in metaphase I and anaphase I (Sapre and Deshpande 1987), and in second meiotic division, generally with lower frequency in the latter case than in first division. Moreover, in some species cytomixis was present with equal or different intensity in all phases of meiosis (de Souza and Pagliarini 1997). In all the alfalfa plants analyzed, we observed cytoplasmic channels and chromatin transfer almost exclusively between meiocytes in the same phase of meiotic division (Figure 1e,d,g,h), as seen in many other species. In addition to cytomixis, still other meiotic abnormalities were observed in the alfalfa plants in both first and second meiotic divisions. These included univalents, laggard chromosomes, chromatin bridges in anaphase I and II, pentads, and chromosome stickiness (Figure 1i).

Unlike the alfalfa mother plants and their S1 and F1 progenies, the control plants of cvs. Adriana had an almost regular meiosis (Figure 1j), and values of cytomixis ranging from 0.5% to 1.6% (mean 1.1%) (Table 1). Adriana 2, the control plant with the lowest percentage of cytomictic cells (0.5%), was crossed with the cytomictic plants to obtain the F1 progenies. The occurrence of cytomixis was analyzed together with pollen viability (see Table 1). All of the mother plants were characterized by a high number of cytomictic microsporocytes and low pollen viability (Figure 1k). The control plants had a much lower frequency of cytomixis (1.1%) and higher pollen viability (94.2%) (Figure 1l) compared with the mean values of the mother plants (45.9% and 54.1% for cytomixis and pollen viability, respectively). Cytomixis has also been reported to occur in different plants from normal populations of other plant species, though in widely variable degrees ranging from very low values—the most frequent case—such as 0.06–2.55% in Brassica campestris and Brassica napus (de Souza and Pagliarini 1997), to values as high as 41.42% for Vigna glabrescens (Seth and Bhattacharyya 1988) and 97.0% for Prunus amygdalus (Soodan and Waffai 1987).

The self progenies inherited cytomixis and can also be considered cytomictic because their levels of cytomixis were significantly different from the 1.1% mean value of the cv. Adriana. All the self progenies had about the same low levels of pollen viability as the mother plants. When the mother plants were crossed with a normal plant (Adriana 2), the trait “cytomixis” segregated in the F1 progenies, because each cross yielded both plants exhibiting little or no cytomixis and plants with high levels of cytomixis. As a result, the average level of cytomixis was lower in the F1 progenies than in their cytomictic parents, probably because of the contribution of the noncytomictic parent. For example, plant Lodi 4 had 36% cytomictic microsporocytes, but the cross Lodi 4 × Adriana 2 had a mean value of cytomixis of only 13.2%. Moreover, no differences in cytomixis values were observed between reciprocal crosses, suggesting that there was no maternal effect. All the crosses showed increased pollen viability compared with the cytomictic parent, indicating a probable contribution of the noncytomictic parent to the F1 progenies, and further suggesting that in M. sativa, cytomixis and pollen viability are associated. However, no significant correlation was found when we calculated the correlation index between the mean values of cytomixis and pollen viability in both the mother plants and the S1 and F1 progenies. Previous authors have found a strictly inverse correlation between cytomixis and pollen viability in some species (Sapre and Deshpande 1987), while in other species, cytomictic plants had high pollen viability (Falistocco et al. 1995). It is reasonable to believe that, besides cytomixis, the other meiotic abnormalities we observed both in first and second meiotic divisions may have adversely affected pollen viability. This would explain why cytomixis alone is not significantly correlated but only associated with pollen viability. Furthermore, no differences in pollen viability were observed between reciprocal crosses.

In order to verify a possible environmental effect on cytomixis, cytomixis and pollen viability of the mother plants were evaluated in two distinct areas of Italy (Perugia and Lodi), characterized by different environments (data not shown). In the two areas, the mother plants showed the same values of cytomixis and pollen viability, while the values of both characters were widely different from those of the cv. Adriana control plants. The results clearly showed that the mother plants were cytomictic irrespective of field location, suggesting that in those plants cytomixis and pollen viability are scarcely influenced by the environment. This view is further supported by the observation that the percentage of cytomixis was similar in the two sets of samples collected in May and September both at Perugia and Lodi (data not shown), in contrast with Basavaiah and Murthy (1987), who found that in Urtica dioica high levels of cytomixis could only be detected in summer.

In conclusion, we have characterized some cytomictic alfalfa plants and our results indicating a lack of environmental effect on cytomixis, its occurrence during a definite phase of meiosis (prophase I), and its persistence in S1 and F1 progenies, all seem to suggest that cytomixis is very likely a natural phenomenon under direct genetic control, as proposed by Mantu and Sharma (1983), although physiological factors certainly influence its manifestation.

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