Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Egizia Falistocco (2022). Polyploid cytotypes and formation of unreduced male gametes in wild and cultivated fennel (*Foeniculum vulgare* Mill.). *Caryologia* 75(3): 85-90. doi: 10.36253/caryologia-1804

Received: August 31, 2022

Accepted: November 22, 2022

Published: April 5, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Polyploid cytotypes and formation of unreduced male gametes in wild and cultivated fennel (*Foeniculum vulgare* Mill.)

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Abstract. *Foeniculum vulgare* Mill. (2n=22) is an herbaceous species native to the Mediterranean region and naturalized in many temperate areas around the world. It includes subsp. *piperitum* and subsp. *vulgare* which are, respectively, the wild and cultivated forms. Fennel is of economic importance both as a vegetable crop and for its wide use in the food and pharmaceutical industries. In recent years, the therapeutic and pharmacological potential of this species has been widely analyzed, its cytogenetic traits have aroused less interest. Therefore, the intention of this study was to reduce this gap by investigating some aspects, such as the variations in its chromosome number and the occurrence of polyploidization events, so far neglected. By means of extensive chromosome counting, the presence of tetraploid cytotypes has been discovered both in the wild and cultivated fennel. Moreover, the analysis of pollen and PMCs at the tetrad stage provided evidence for spontaneous sexual polyploidization as the most probable origin of the tetraploid cytotypes discovered. The results of this study provide the first evidence of the occurrence of polyploidization events in *F. vulgare* and suggest that the use of 2*n* gametes could be a useful approach to genetic improvement of this crop.

Keywords: *Foeniculum vulgare* Mill., fennel, polyploid cytotypes, polyploidization, unreduced gametes.

INTRODUCTION

Foeniculum vulgare Mill., of the Apiaceae (syn. Umbelliferae) family, is a biennial-perennial herb native to the Southern Mediterranean region from where it was brought for cultivation throughout the temperate regions of Asia, North America and Europe. Fennel is described as a diploid species (2n=22) characterized by erect, cylindrical, bright green and smooth stems growing up to 2 m in height. Its leaves are finely dissected with terminal filiform segments. Its bright yellow flowers are contained in terminal compound umbels with a variable number of rays (Subramanian 1986; Badgujar 2014).

Two subspecies of *F. vulgare* are generally recognized: subsp. *piperitum* and subsp. *vulgare* which represent the wild and cultivated forms, respec-

tively. The wild fennel (subsp. *piperitum*) is abundantly present in the Mediterranean flora and commonly found in limey soil near the sea and on river banks. Subsp. *vulgare* includes var. *azoricum* which is cultivated as a vegetable for its enlarged leaf base and var. *dulce* which is grown prevalently for its seeds containing essential oil (Pignatti 1982).

F. vulgare is commonly called fennel and it is denoted locally by more than 100 vernacular names. It is a traditional and popular species with a long history having been appreciated and utilized as a medicinal, aromatic and edible plant at least since Roman times up to the present day. In some countries, for example, fennel is currently used to stimulate lactation and to improve the taste of some medicines, while the seeds are widely used as a spice (Simon et al. 1984; Sheidai et al. 2007; Faudale et al. 2008; Badgujar et al. 2014; Rather et al. 2012).

F. vulgare is at present a species of considerable economic interest not only for its wide diffusion as a vegetable crop but also for its pharmaceutical properties which, in recent years, have been re-evaluated and extensively investigated. The species possesses antioxidant, antispasmodic, carminative, diuretic and laxative properties and is useful in the treatment of numerous infectious disorders of bacterial, fungal, viral and protozoal origin (Agarwal et al. 2017). Fennel is also widely used in the food industry as a flavor additive to meats, salad dressing, breads, pastries, teas and alcoholic beverages (Badgujar et al. 2014).

Despite its economic importance and wide diffusion in the wild, only limited attention has been dedicated to it by cytologists mainly interested in its karyological aspects (Garde and Garde 1949; Das and Mallick 1988; Lentini et al. 1988; Paul and Datta 2003¸ Subramanian 1986; Jovine et al. 2008; Ozkan et al. 2017). Most of these studies were carried out on only few samples. Extensive cytogenetic investigations have been to date very limited. The purpose of this study was to expand the study of *F. vulgare* and explore areas of cytogenetics so far overlooked, such as the existence of chromosome variants and events of polyploidization. To realize this, investigations on wild and cultivated populations of *F. vulgare* were carried out by attempting, as first step, an extensive chromosome count. Then, because tetraploid cytotypes were identified during this survey, the successive objective was to clarify the contribution of 2n gametes to the origin of the polyploid plants discovered. For this purpose the size of pollen grains and the constitution of sporads were analyzed to verify the occurrence of unreduced gametes and to estimate the frequency of their formation. It has been widely demonstrated that pollen grains and PMCs (Pollen Mother Cells) at the tetrad stage provide essential data on the tendency of plants to produce 2n male gametes and offer information on the origin of polyploids (Orjeda et al., 1990; Ramsey and Schemske 1998; Garcia et al. 2020). Large pollen is normally 2*n* pollen because there is a positive correlation between DNA content and cell volume, which in turn influences the size of the pollen grain. Thus, 2n pollen grains are larger than reduced grains. On the other hand, microsporogenesis offers irrefutable indications of the occurrence of 2n gametes because the presence of dyads and triads in the sporad stage constitutes strong evidence for the formation of unreduced pollen (Garcia et al. 2020). This study is the first to document the occurrence of polyploidization events in *F. vulgare*. It also provides data supporting sexual polyploidization as the possible origin of the polyploid cytotypes discovered.

MATERIALS AND METHODS

Plant material

Seeds and plants of *F. vulgare* subsp. *piperitum* and subsp. *vulgare* were used for this study.

Seeds of wild fennel (subsp. *piperitum*) were collected by the author in two different localities of Italy: the countryside surrounding the Trasimeno Lake in Central Italy (population1), and the North-East coast of Sardinia (population 2). Seeds of the cultivated varieties *azoricum* and *dulce* (subsp. *vulgare*) were provided by specialized nurseries (Table 1).

The seeds were germinated at 20-22 °C in Petri dishes on filter paper moistened with distilled water. A part of the seedlings was transplanted to obtain plants for the production of flowers and roots. A total of 400 seeds and 10 plants for each accession were checked for their chromosome numbers. The same plants were also used for the analysis of microsporogenesis and pollen size.

Chromosome counts

Roots 5-10 mm long were collected from seedlings and adult plants and immersed in ice-cold water for about 16 h to accumulate metaphases. Then they were pre-treated in a 1‰ aqueous solution of a stock solution consisting of 1 ml of a-bromonaphthalene dissolved in 100 ml of absolute ethanol (Linde-Laursen 1978) for 3 h, fixed in ethanol-glacial acetic acid (3:1) at room temperature overnight and then stored at -20 °C until required. Mitotic chromosome preparations were realized according to the protocol described in detail in Falistocco (2018).

Table 1. List of accessions of *F. vulgare* subspp. *piperitum* and *vulgare* examined and number of tetraploid (2n=44) plants discovered.

Subspecies	Accessions	Origin	n. of tetraploid (2n=44) plants
piperitum	population 1	Central Italy	3
	population 2	Sardinia	4
vulgare	cv. *azoricum*	commercial source	5
	cv. *dulce*	commercial source	7

The excised roots were washed in distilled water for 10 min and transferred to the enzyme buffer (10mM citric acid/sodium citrate, pH 4.6) for 20 min. Root tips were then excised and digested in the enzyme solution (4% cellulase Onozuka R10 and 1% pectolyase Sigma-Aldrich in distilled water) for 45-60 min at 37°C. The cell suspension was pelleted and resuspended in enzyme buffer. After pelleting, the material was washed twice with the fixative and resuspended in the fixative. Finally, 20-30 ml of cell suspension was applied to a slide. The slides were air-dried and stained with 2mg/ml DAPI (4', 6-diamidino-2-phenylindole) for the determination of the chromosome numbers.

Detection of unreduced male gametes

Pollen analysis

Pollen from five flowers per plant was spread over five slides and stained with a solution of acetocarmine and glycerol (1:1). The dark colored and regular shaped pollen grains were considered as viable. The pollen size was determined by measuring the major diameter of the grains by using an ocular micrometer. Measurements revealed two types of pollen grains which can be considered normal pollen and large pollen. The size of the normal pollen was determined by measuring the grains present in three light vision fields of the microscope for each slide. About 600 grains for each plant were measured. All large pollen grains present in the slides were measured.

Analysis of PMCs at the tetrad stage

Inflorescences were collected and immersed in the fixative ethanol-glacial acetic acid (3:1) for 24 hours and then they were transferred to 70% ethanol and stored at 4°C until analysis. For cytological preparations anthers of a single flower were squashed on a glass slide with some drops of 0.5% acetocarmine (Merk Life Science, Italy), intensified by ferric oxide. In order to select only anthers containing PMCs at the tetrad stage, preliminary observations were made to assess the meiotic stage of the flowers. One single anther was removed from the floral bud, squashed on a slide as described above and examined. When the anther contained sporads the other anthers of the same flower were used for cytological preparations. Five flowers per plant were analyzed. The number of dyads, triads and tetrads detected in four light vision fields for each slide were counted. About 1500 sporads for each plant were examined. Estimation of the theoretical frequency of 2n pollen grains was made from the number of observed dyads, triads, and tetrads at the end of microsporogenesis. Considering that a dyad evolves into two unreduced pollen grains, a triad produces one unreduced pollen grain and two reduced pollen grains, and each tetrad gives origin to four reduced pollen grains, the frequency of 2n pollen grains was calculated by applying the equation F2n (%) = (2xDy + Tr) / (2xDy+ 3x Tr + 4x Te) x 100, for which Dy, Tr and Te, are the number of dyads, triads and tetrads, respectively (Kumar and Singhal 2012).

Chromosome preparations and slides containing pollen and sporads were observed under UV and light illumination, respectively, with a Microphot Nikon microscope. Images were recorded with a digital photocamera SONY ICX282AQ and then processed using Adobe Photoshop 5.0.

RESULTS

Chromosome counts

All plants examined resulted diploid having the chromosome number 2n=22 (Figure 1a), but polyploid seedlings were detected in each accession of subspp. *piperitum* and *vulgare* (Figure 1b). Three seeds from population 1 (Central Italy) and four seeds from population 2 (Sardinia) were found to be tetraploid with 2n=2x=44 (Figure 1b). The frequency of tetraploids discovered in cultivated fennel was greater: five in the cv. *azoricum* and seven in the cv. *dulce* (Table 1). The tetraploid condition was always clearly distinguishable in all metaphases analyzed. No other ploidy levels were detected.

Estimation of the occurrence of unreduced male gametes

To detect the formation of unreduced male gametes pollen size was measured and the constitution of the sporads analyzed.

Full pollen viability was generally observed, with very sporadic cases of shriveled and unstained grains present.

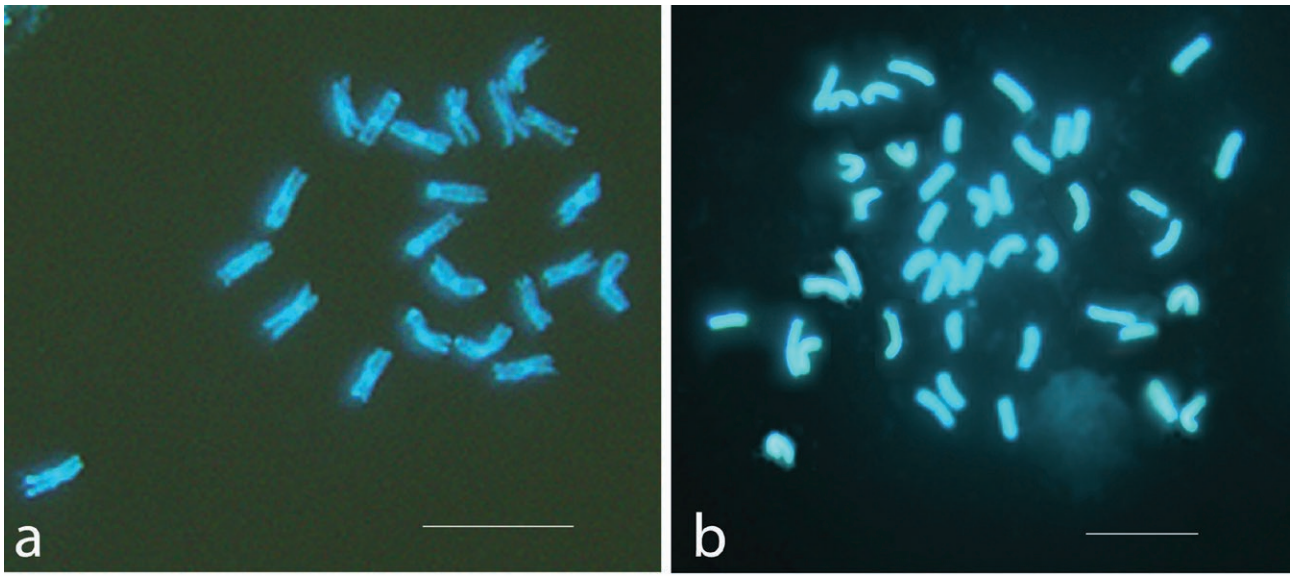

Figure 1. Mitotic metaphases of *F. vulgare*. Chromosome complements of diploid (a) and tetraploid (b) cytotypes. The bar represents 5µm.

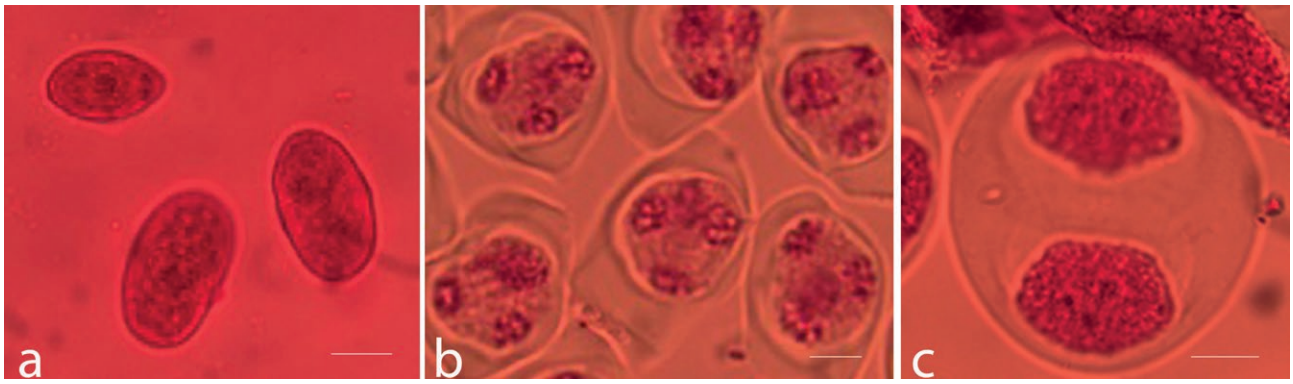

Figure 2. Example of pollen grains and PMCs at the sporad stage observed in plants producing large pollen. Pollen sample showing normal and large pollen grains (a); group of tetrads with four *n* (reduced) microspores (b); dyad containing two 2*n* (unreduced) microspores (c). The bar represents 10 µm.

Most of plants examined produced pollen of uniform size, but few plants produced also noticeably larger grains (Figure 2a). Diameter measurements confirmed the results of visual observation. According to their size, pollen grains were categorized as *n*, that is normal reduced pollen, with its diameter ranging from 33.0 to 35.0 µm; and 2*n*, unreduced pollen, measuring from 42.0 to 44.0 µm. Large pollen was identified as unreduced 2n pollen according to Darlington (1937) who defines as 2n pollen the grains with a size 1,25x larger than the average size of normal pollen. Pollen grains of intermediate size were not observed. Large pollen grains were detected in two plants of population 1, three plants of population 2, one plant of cv. *azoricum* and three plants of cv. *dulce* (Table 2). The sporad constitution was analyzed to confirm that large pollen grains effectively represent 2*n* pollen production. This analysis revealed, in addition to the expected normal tetrads, the presence of dyads in all plants producing large pollen grains (Figure 2b,c). The frequency of dyads produced was 2.26 and 4.0% in plants of population 1; 2.44, 3.0 and 4.0% in plants of population 2; 5.0% in the plant of var. *azoricum*; and 5.00, 7.00 and 8.00% in plants of var. *dulce* (Table 2). Dyads or triads were not observed in the remaining plants examined. Additionally, abnormal tetrads (that is containing microspores of different sizes, collapsed or with micronuclei) or polyads were not found in any of the plants object of this analysis. By the rule that each tetrad can form four *n* micro-

Table 2. Frequencies of dyads and 2*n* pollen recorded in plants of of subspp. *piperitum* and *vulgare* producing large pollen.

Subspecies	Accessions	Plants * producing large pollen	Dyad %	2n pollen %
piperitum	population 1	3	2.26	1.14
		7	4.00	2.00
	population 2	1	2.44	1.23
		4	3.00	1.50
		9	4.00	2.00
vulgare	cv. *azoricum*	2	5.00	2.50
	cv.*dulce*	3	5.00	2.50
		5	7.00	3.60
		7	8.00	4.10

*Plants examined were numbered from 1 to 10.

spores and each dyad can form two 2*n* microspores, the frequency of 2*n* pollen grains could be estimated for each plant according to the abovementioned formula. The results are reported in Table 2.

DISCUSSION

The discovery of tetraploid cytotypes deriving from this study provided the first evidence of polyploidy in *F. vulgare*. Furthermore, the pollen and sporad analyzed indicate spontaneous sexual polyploidization as the most probable origin of such cytotypes. Various methods exist to reveal the formation of 2*n* pollen and one of these is based on the size of the pollen grains. Due to the relatively close correlation between large pollen and the 2*n* status, the presence of large pollen has been frequently used as an indicator for 2*n* pollen (Ghaffari 2006; Kumar and Singhal 2012). The analysis of pollen size carried out during this study revealed two types of pollen grains which according to the criterion of Darlington (1937) were classified as *n* (normal reduced) and 2*n* (unreduced) pollen. Further evidence that large pollen effectively indicates unreduced pollen formation was provided by the sporad analysis demonstrating that fennel plants producing dyads also produce large pollen grains. The large 2n pollen grains examined were well filled, stained, and apparently fertile; therefore, it is very possible that fertilization by these 2n gametes led to the formation of polyploid cytotypes. The tetraploid constitution of these cytotypes suggests that in fennel unreduced 2n gametes are generated also during the macrosporogenesis process.

The formation of 2n pollen grains in *F. vulgare* has been previously observed in natural populations from Iran (Sheidai et al. 2007); but they have never been sought in cultivated fennel. The production of 2n gametes in plants is a common phenomenon which may result from a variety of different meiotic irregularities (Dewitte et al. 2012). The microsporogenesis analysis carried out during this study was focused on meiocytes at the tetrad stage, so that the meiotic events occurring in the phases preceding the tetrad stage still remain unknown. Therefore, no definitive conclusion on the meiotic aberrations responsible for the formation of 2n pollen is possible. The absence of abnormal sporads and the irrelevant number of non viable pollen grains observed would exclude the occurrence of meiotic abnormalities affecting chromosome segregation with the consequent formation of aneuploid gametes. Rather, the regularity of microspores would suggest that the origin of unreduced pollen is principally due to the meiotic events connected to a process of nuclear restitution during the first or second division.

Another interesting point is the higher incidence of tetraploids detected in the cultivars with respect to wild populations. The greater tendency of cultivated plants to produce unreduced gametes could be connected to this phenomenon. However, it may be assumed that the tetraploid condition generates characteristics in plants favoring their selection during the breeding activities.

Clarification of this and the other hypotheses inherent in this study will come from further investigations. The fact that tetraploid plants were found in all accessions examined suggests that polyploidy in *F. vulgare* is a rather widespread phenomenon and not a sporadic event. The demonstrated characteristic of fennel plants to produce 2n gametes should be exploited to generate polyploid genotypes. The induction of polyploidy could be a useful method for improving specific traits in crop varieties, such as quality, yield and environmental adaptation. In fennel, this practice has been attempted by colchicine treatments but with scarce results (Solanki et al. 2017). Polyploid plants obtained by sexual polyploidization may turn out to be a promising approach to breeding programs of this species.

FUNDING

This work was supported by Fondazione Cassa di Risparmio di Perugia (Italy), project code: 2019.0317.029.

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