

## Unreduced gametes in diploid *Medicago* and their importance in alfalfa breeding\*

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**Summary.** In the genus *Medicago*, it is known that 2n gametes have been important in the evolution and breeding of cultivated alfalfa, which is a natural polysomic polyploid ( $2n=4x=32$ ), however little is known on the frequency of male and female 2n gametes in diploid relatives of alfalfa. To obtain data on the frequency of 2n gametes, more than 12,000  $2x-4x$  and  $4x-2x$  crosses were made in 1982 at Madison (USA). Diploid parents in crosses were from four populations of *M. coerulea*, two of *M. falcata* and one diploid population of cultivated *M. sativa* which was derived by haploidy. The tetraploid seed parent in the crosses was a male-sterile *M. sativa* clone and vigorous tetraploid *M. sativa* plants were used as pollen parents. Each of 274 diploid plants was utilized both as male and as female. Of the 548 cross combinations, 266 crosses produced variable quantities of seeds which were sown in 1983 in a greenhouse at Perugia (Italy); the plants were subsequently space transplanted in the field in 1984. The identification of ploidy level of these genotypes was made on the basis of morphological characters, plant fertility, pollen stainability and chromosome counts.

Of the 515 plants analyzed, the majority behaved as normal tetraploids indicating that many diploid plants produced 2n gametes. Diplogynous and diplandrous gamete production was not correlated with each other, which indicated a different genetic control of 2n sporogenesis in the 2 sexes. Only 4  $F_1$  triploid plants con-

firmed the presence of a very effective triploid block in alfalfa. In consequence, bilateral sexual polyploidization is a more likely alternative for the origin of tetraploid alfalfa than triploid bridges. The present study showed that it is possible to efficiently identify genotypes able to produce high frequencies of 2n gametes within natural populations of diploids *Medicago* that are useful in alfalfa breeding.

**Key words:** Alfalfa – *Medicago* genus – Polysomic polyploids – Sporogenesis – Unreduced gametes

### Introduction

Gametes with the unreduced chromosome number (herein termed 2n gametes) have been reported to occur naturally at a low frequency in a number of plant species: *Datura* (Satina and Blakeslee 1935), *Dichanthium* (deWet and Harlan 1970), *Fragaria* (Bringham and Gill 1970), *Medicago* (Bingham 1969), *Pisum sativum* (Myers et al. 1984), *Solanum* (Quinn et al. 1974), *Zea mays* (Rhodes and Dempsey 1966). They most likely play a major role in the evolution of polyploid series (Harlan and deWet 1975).

In *Medicago* genus, several authors have stressed the importance of 2n gametes both in the evolution (Stanford et al. 1972) and breeding (Bingham 1968, 1979; Vorsa and Bingham 1979; McCoy and Smith 1983) of cultivated alfalfa, which is a natural polysomic polyploid ( $2n=4x=32$ ). Alfalfa performance appears to be dependent on heterozygosity and, particularly, on the frequency of tri- and tetra-allelic loci (Demarly 1963; Busbice and Wilsie 1966; Dunbier and Bingham 1975). Consequently, the main target of the breeder in developing new cultivars is to maximize heterozygosity.

In a cross-fertilizing natural polyploid such as alfalfa heterozygosity can be maximized but not fixed by normal

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sexual means (Dunbier and Bingham 1975). An alternative to normal sexual reproduction could be a non-conventional approach characterized by employing alterations of the sexual cycle with the hybridization of 2n gametes from diploid hybrids. Lewis and John (1963) and Rhoades and Dempsey (1966) have enumerated the ways in which 2n gametes could conceivably arise. These include premeiotic doubling, first division restitution (FDR), normal first division followed by chromosomal replication during interphase and a normal second division, second division restitution (SDR), and postmeiotic doubling.

In particular, 2n gametes produced by FDR or an equivalent process can be an effective breeding tool in polysomic polyploids, transmitting all of the heterozygosity from the centromere to the first crossover and half of that between the first and the second crossover (Mendiburu 1971; Mock and Peloquin 1975).

In species such as alfalfa, where there is often only one chiasma per bivalent, it is estimated that approximately 80% of the heterozygosity is transmitted from parent to offspring via FDR 2n gametes. The ultimate use of 2n gametes in maximizing heterozygosity in alfalfa would be, therefore, the union of FDR-type 2n eggs and FDR-type 2n pollen from hybrid diploid parents to produce a sexual polyploid (Bingham 1979). Furthermore, 2n gametes are also important in alfalfa for unidirectional genetic introgression by interploid crosses from wild diploids to the cultivated tetraploid level (Bingham 1968; Stanford et al. 1972; Barnes et al. 1977).

In cultivated alfalfa at the diploid level (CADL), which was developed from the cultivated tetraploid by haploidy, several diploid plants have been identified which produce a high frequency of 2n male gametes (Bingham and McCoy 1979). Vorsa and Bingham (1979) reported that 2n pollen formation in these plants was caused by a disorientation of spindles at metaphase II, and that the 2n gametes were genetically equivalent to FDR. McCoy (1982) concluded that 2n pollen formation in one of the plants was controlled by a single recessive gene with variable expression.

Much less information is available on 2n egg producers in alfalfa. Pfeiffer and Bingham (1983) reported cytological and genetic evidence which indicated that 2n egg formation in two plants (one 2x and one 4x) was due to SDR. The SDR mode of 2n gamete formation theoretically transmits less heterozygosity than FDR (Peloquin 1979) and may be less useful in breeding.

On the whole, little data is available on the frequency of male and female 2n gametes in natural diploids belonging to the *Medicago sativa-coreulea-falcata* complex. The present study was conducted to determine the frequency of male and female 2n gametes in eight diverse diploid populations in the complex and to identify genotypes able to produce high frequencies of 2n gametes for use in breeding programs.

## Materials and methods

To obtain data on the frequency of 2n gametes, 12,437 2x–4x and 4x–2x crosses were made by hand in the greenhouse during autumn 1982 at Madison, Wis, USA. At the diploid level, crosses involved five accessions of *M. sativa* subsp. *coerulea* (Lessing ex Ledebour) Schmalhansen, two accessions of *M. sativa* subsp. *falcata* (L.) Arcangeli and one population of cultivated alfalfa (herein termed as CADL; Wisconsin seed lot 2x-iso-1) developed from a cultivated tetraploid using haploidy with breeding and selection (Bingham and McCoy 1979). At

the tetraploid level, a male-sterile clone (6–4 ms) and several vigorous tetraploid *M. sativa* plants were utilized.

The 7 natural diploid accessions were kindly supplied by the Regional Plant Introduction Station, Iowa State University, Ames, Iowa, USA. The number of plants used for each diploid population ranged from 14 (*M. coerulea* 325381) to 79 (*M. sativa* CADL).

Each one of the 274 diploid plants available was utilized both as a male, on 6–4 ms, and as a female, receiving pollen from tetraploid *M. sativa* plants. For each diploid plant at least 10 flowers were pollinated on 6–4 ms and 10 flowers were pollinated by pollen coming from 4x plants. Each day that crosses were to be made, tetraploid plants were intercrossed as controls. The 1,454 seeds produced in 2x–4x and 4x–2x crosses were sown in a greenhouse at Perugia (Italy) in spring 1983 and the surviving plants were space transplanted in the field in spring 1984.

During 1983–84 a preliminary identification of the progeny ploidy level was made on the basis of morphological characters, pollen stainability and plant fertility (evaluated by crosses with vigorous *M. sativa* tetraploid plants under isolation cages to prevent contamination). The plants which did not show a clear ploidy level on the basis of the preliminary identification were checked cytologically during 1985. Rooted cuttings from these plants were used to make root-tip chromosome counts. Root tips were pretreated in saturated monobromonaphthalene for 5 h, then fixed in 3:1 (ethanol: acetic acid) overnight and stored in 70% ethanol. Root-tip squashes were stained by the Feulgen technique. The counts were made on about 20 metaphases for each plant. The ploidy level of 86 plants was determined cytologically.

## Results

The percentage of diploid plants which produced seeds in interploidy crosses (Table 1) ranged between 0% (*M. coerulea* 325381) and 51% (*M. coerulea* 243225) when utilized as pollen sources and between 46% (*M. falcata* 258754 and *M. sativa* CADL) and 100% (*M. coerulea* 325381 and 243225) when utilized as eggs sources. Extremely variable quantities of seed were produced by 266 out of 548 cross combinations.

Seeds set after interploid crosses was used as a rough measure of 2n gametes production, as had already been done in *Medicago* by Bingham and McCoy (1979).

Average seed set ranged between 2% (*M. coerulea* 315462, 4x–2x crosses and *M. coerulea* 315465, 4x–2x crosses) and 30% (*M. sativa* CADL, 4x–2x crosses) except for 4x–2x crosses involving *M. coerulea* 325381 which failed to produce seeds.

Production of 2n gametes was widespread within the diploid populations. As reported in the last line of Table 1, within each cross combination it was possible to find at least one plant characterized by seed set far higher than the average value of the population.

Ploidy level identification was possible for 515 plants which, coming from the 1,454 seeds produced in the 2x–4x and 4x–2x crosses, survived during the period 1983–85. Results of this analysis are reported in Table 2. As it was expected, in the 4x–2x category al-

**Table 1.** Number of 2x plants utilized, percentage of 2x plants producing seeds and fertility information relative to interloid crosses

	<i>M. coerulea</i>				<i>M. falcata</i>				<i>M. sativa</i>							
	(315466) USSR 4x-2x 2x-4x	(315462) USSR 4x-2x 2x-4x	(325381) USSR 4x-2x 2x-4x	(315465) USSR 4x-2x 2x-4x	(243225) Iran 4x-2x 2x-4x	(262532) Israel 4x-2x 2x-4x	(258754) USSR 4x-2x 2x-4x	(258754) USSR 4x-2x 2x-4x	CADL 2x-iso-1 4x-2x 2x-4x							
Total no. of 2x plants utilized	24	30	14	23	39	32	33	79								
Percentage of 2x plants producing seeds	42	83	27	70	0	100	35	74	51	100	34	50	9	46	37	66
Total no. of flowers pollinated	501	726	581	917	251	536	439	672	778	1,263	581	647	571	1,094	1,250	1,630
Total no. of seeds produced	13	138	12	62	0	71	11	61	29	319	15	38	3	141	374	167
Average seed set (%)	3	19	2	7	0	13	2	9	4	25	3	6	0.5	13	30	10
Maximum seed set (%)	12	123	15	32	0	32	15	29	17	78	15	36	7	118	600	96

**Table 2.** Number of plants analyzed and of tetraploid plants detected in interloid crosses

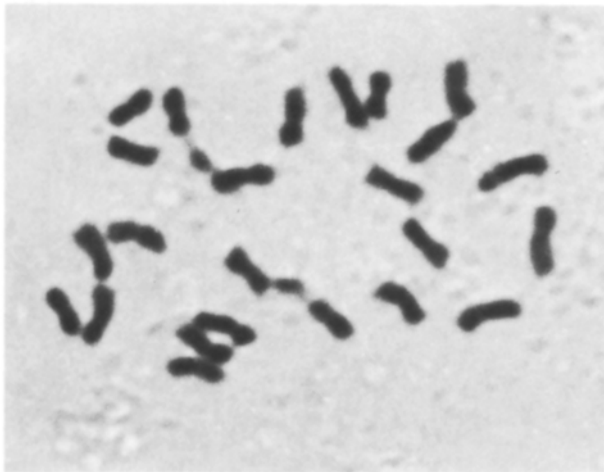
	<i>M. coerulea</i>				<i>M. falcata</i>				<i>M. sativa</i>							
	(315466) USSR 4x-2x 2x-4x	(315462) USSR 4x-2x 2x-4x	(325381) USSR 4x-2x 2x-4x	(315465) USSR 4x-2x 2x-4x	(243225) Iran 4x-2x 2x-4x	(262532) Israel 4x-2x 2x-4x	(258754) USSR 4x-2x 2x-4x	(258754) USSR 4x-2x 2x-4x	CADL 2x-iso-1 4x-2x 2x-4x							
No. of plants analyzed	3	20	4	11	-	11	4	24	10	126	1	14	-	69	157	61
No. of 4x plants detected	3	2	4	7	-	7	4	14	9	28	1	11	-	10	155	52
% of 4x plants*	100.0	10.00	100.00	63.64	-	63.64	100.00	58.33	90.00	22.22	100.00	78.57	-	14.49	68.73	85.25

\* % of 4x plants = (no. of 4x plants detected)/(no. of plants grown to maturity) x 100

**Table 3.** Correlation coefficients ( $r$ ) between the number of tetraploid  $F_1$  plants obtained utilizing each diploid plant both as male and as female

Diploid populations	Correlation coefficients ( $r$ )
<i>M. coerulea</i>	
315466	-0.3273 ns (n = 10)
315462	-0.2785 ns (n = 12)
325381	(see Table 2)
315465	-0.0690 ns (n = 12)
243225	-0.0174 ns (n = 35)
<i>M. falcata</i>	
262532	-0.2652 ns (n = 12)
258754	(see Table 2)
<i>M. sativa</i>	
CADL	-0.0254 ns (n = 30)

ns = not significant



**Fig. 1.** Triploid  $F_1$  plant (*M. sativa* 6-4 ms  $\times$  *M. coerulea* 243225)

most all the plants were tetraploid while a certain amount of diploids were detected when  $2x$  plants were used as female parents. *M. coerulea* 243225 was characterized by quite an high level of self-fertility as shown by 98 diploids out of 126 plants analyzed. On the whole, ploidy level identification confirmed the presence of  $2n$  gametes in all the diploid populations. Only four triploid plants (Fig. 1) were identified, one produced in  $2x-4x$  crosses with *M. coerulea* 315466, one in  $4x-2x$  crosses with *M. coerulea* 243225 and two in  $4x-2x$  crosses with CADL.

Correlation coefficients ( $r$ ) between the number of  $4x$  plants obtained utilizing each diploid plant both as male and as female are reported in Table 3. Due to the absence of any significant correlation, at the single plant level  $2n$  pollen production did not appear to influence

$2n$  eggs and vice versa. The absence of correlation confirms also that there were no undetected  $4x$  plants among the diploid plants used in crosses made in Madison, Wis.

## Discussion

Results of the interploidy crosses in the *Medicago sativa-coerulea-falcata* complex suggest that  $2n$  gametes are prevalent in natural diploid populations as well as in the derived diploid CADL population. Diplogynous and diplandrous gamete productions were not correlated with each other in any population, which seems to indicate a different genetic control of  $2n$  sporogenesis in the 2 sexes.

The presence of  $2n$  pollen and eggs provides the natural diploid populations with the potentiality of undergoing a bilateral sexual polyploidization (BSP) process through  $2x-2x$  matings with the production of tetraploid progeny. The occurrence of BSP in CADL was discussed by Bingham and McCoy (1979) and was recently reported in red clover by Parrott and Smith (1985).

The identification of only 4  $F_1$  triploid plants confirms the presence in alfalfa of a very effective triploid block which, as already pointed out in several species by Brink and Cooper (1947); Hanneman and Peloquin (1968); Von Wangenheim et al. (1960) and Johnston et al. (1980), operates in interploidy crosses eliminating almost all triploid embryos due to endosperm imbalances. As a consequence, in alfalfa, BSP is a more likely alternative to the triploid bridge hypothesis proposed by Harlan and deWet (1975) with respect to the origin of polyploids in angiosperm. Moreover, the widespread occurrence of  $2n$  gametes in *M. coerulea* and *M. falcata*, which are strongly related to cultivated alfalfa, supports the possibility of continuous introgression from diploid to tetraploid forms via  $2n$  pollen within the *Medicago sativa-coerulea-falcata* complex.

It is apparent from the results of this study and others (Bingham and McCoy 1979; Vorsa and Bingham 1979; McCoy 1982; Pfeiffer and Bingham 1983) that diploid plants with a high frequency of  $2n$  eggs and pollen can be identified in wild relatives of alfalfa as well as in CADL. Furthermore, due to the absence of correlation between unreduced gamete production in the two sexes shown in the present study, it seems feasible to obtain plants characterized by production of  $2n$  pollen and  $2n$  eggs, respectively. The next step could be the increase of  $2n$  gamete frequency through selection, as already done in potatoes by Den Nijs (1977) and proposed in red clover by Parrott et al. (1985). This breeding approach should be very useful in maximizing heterozygosity in alfalfa.

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