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MEIOTIC BEHAVIOUR OF CHROMOSOMES IN PMCS AND KARYOTYPE OF *TRIFOLIUM REPENS* L. FROM DARJEELING HIMALAYA

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Detailed analyses of the chromosome meiotic behaviour and of mitotic metaphase chromosomes (2n = 32), as well as stainability studies of pollen fertility, were carried out in order to determine the cytological status of amphidiploid *Trifolium repens* L. (Dutch white clover). In amphidiploid (allotetraploid) *Trifolium repens* L., diploid-like meiotic behaviour of chromosomes was found, with no multivalent formation, and a normal karyotype with a single pair of chromosome having a secondary constriction was observed. These characteristics indicate favourable genetic and cytological stability in nature, and high pollen fertility further enhances its usefulness in breeding.

Key words: *Trifolium repens* L., meiotic chromosome, karyological analysis.

INTRODUCTION

Cytogenetically, most species of *Trifolium* (white clover), an important forage legume, have a diploid chromosome number of 16. Polyploidy is uncommon in *Trifolium*. Only 16% of the 248 species of *Trifolium* distributed throughout the world are polyploid. However, 70% of the known polyploids occur in the subgenus *Amoria*, which is considered to be one of the most primitive and unspecialised subgenera. Williams (1987) indicated that the polyploid species are often indigenous either to a region far removed from Mediterranean (center of origin) or to a temperate hilly region.

Trifolium repens L. is one such polyploid species in the subgenus *Amoria*. Williams (1987) considered *Trifolium* to be an allotetraploid (2n = 32). Ansari et al. (1999) also supported the allopolyploid origin of white clover. Jones et al. (2003) constructed a framework genetic map of white clover (*Trifolium repens* L.), using an SSR and AFLP molecular markers. The present study attempts to determine the present cytological status of polyploid *Trifolium repens* L. (Dutch white clover) from Darjeeling Himalaya (latitude 27˚2'57"N, longitude 88˚15'5"E; altitude 8000 feet a.s .l.) by analysing the meiotic behaviour of the chromosomes, the karyotype, and pollen fertility.

MATERIALS AND METHODS

For study of meiotic chromosomes, suitable flower buds were collected and fixed overnight in propionic acid and absolute alcohol (propionoalcohol 1:3). Anthers were smeared in 2% propionocarmine stain. Analysis of meiotic chromosomes from pollen mother cells was confined to observations of chromosomes at diakinesis, mean chiasma frequencies, metaphase I and anaphase I. At least 50 suitably scattered meiotic plates were compared to ascertain the number of bivalents and to detect meiotic abnormalities.

Mitotic chromosome counts were made from young and fresh root tips pretreated with a saturated solution of paradichlorobenzene (pDB) for 4 h at 14˚C, fixed in 1:3 propionoalcohol for 1 h at room

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TABLE 1. Results of detailed analysis of karyotype of Dutch white clover (*Trifolium repens* L.). SA – short arm of chromosome; LA – long arm of chromosome; WL – whole length of chromosome; ST – sub terminal primary constriction; SC – secondary constriction; F % – centromeric index; r – arm ratio (long arm/short arm); d – long arm - short arm

Type of chromosome	No.of chromosome	Length of SA (μm)	Length of LA (μm)	WL (μm)	r value	d value	F %	Remarks
A	2	0.262 0.294	1.838 1.806	2.10	7.00 6.00	1.576 1.512	12.50 14.00	ST with SC
B	8	1.050 0.870 0.830 0.650	1.050 0.870 0.830 0.650	2.10 1.74 1.66 1.30	1.00 1.00 1.00 1.00	0 0 0 0	50 50 50 50	Median
C	22	0.517 0.425 0.375 0.350 0.330 0.325 0.320 0.315 0.311 0.310 0.305	1.560 1.276 1.125 1.050 0.990 0.975 0.960 0.945 0.934 0.930 0.915	2.07 1.70 1.50 1.40 1.32 1.30 1.28 1.26 1.24 1.24 1.22	3.00 3.00 3.00 3.00 3.00 3.00 3.00 3.00 3.00 3.00 3.00	1.04 0.85 0.75 0.70 0.66 0.65 0.64 0.63 0.62 0.62 0.61	25 25 25 25 25 25 25 25 25 25 25	Sub-median

TF – total centromeric index = 41.88%; DI – disparity index = 26.50%; MF – mean F % = 1.30%.

temperature. Root tips were kept in 45% propionic acid for 5 min, then warmed in 2% propionoorcein-HCl (1N; 9:1) for 1.5 h at room temperature (18˚C). Root tips were squashed in 45% propionic acid for microscopy. During analysis of karyotypes, 10 to 15 well-spread metaphase chromosome plates were compared and analysed.

To estimate pollen fertility, mature anthers were dehisced over a glass slide to which one-drop of 2% propionocarmine stain was added. After 5 min of staining, the numbers of fully stained grains as well as hyaline grains were counted in the optical field. At least 500 grains from two or more flowers per plant were examined.

RESULTS

The chromosomes of tetraploid *Trifolium repens* L. (Dutch white clover) from Darjeeling Himalaya behave as in a regular diploid at meiosis. Chromosome pairing was very regular at diakinesis. Mean chiasma frequency was 15.83 per cell. Various cells ranged from 14 to 16 chiasmata. Meiotic chromosomes formed the normal 16 bivalents at metaphase I (Fig. 1). Pollen mother cells in anaphase I were also examined to confirm the metaphase chromosome counts. The bivalents segregated regularly, 16–16 separations at anaphase I. There was no irregularity in meiotic cell division. No univalent, multivalent or lagging chromosomes were observed.

Mitosis in root tip cells showed regular cell division. The chromosome number counted from the mitotic metaphase plate was 2n = 32 (Fig. 2). In general, the chromosomes were short (maximum length 2.10 μm) and bi-armed. On the basis of centromere position, the chromosomes were identified and classified into three types according to Levan et al. (1964) (Tab. 1, Fig. 3):

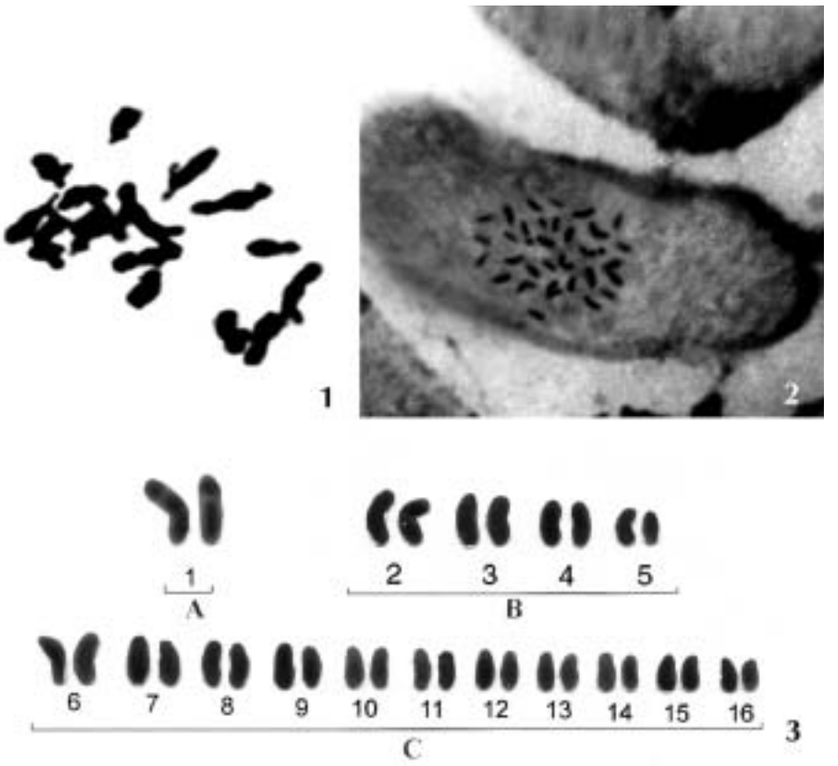
Type A: Chromosomes 2.10 μm in length, with two constrictions, one primary and the other secondary; 2 chromosomes, subtelocentric type.

Type B: Chromosomes between 1.30 μm and 2.10 μm, with one constriction, primary constriction located at median position; 8 chromosomes, metacentric type.

Type C: Chromosomes between 1.22 μm and 2.07 μm, with one constriction, primary constriction located at submedian position; 22 chromosomes, submetacentric type.

Karyotype formula = $A_2 + B_8 + C_{22}$.

The fertile pollen grains were comparatively large, with deeply stained cytoplasm. Sterile grains



Figs. 1–3. *Trifolium repens* L. Mitotic and meiotic chromosomes. **Fig. 1.** Metaphase I with 16 bivalents. × 760. **Fig. 2.** Mitotic metaphase (2n = 32). × 1650. **Fig. 3.** Chromosome types in karyotype.

were hyaline, as they had no cytoplasm. The mean percentage of pollen stainability was 95.8%, and the range was 93.7% to 97.9%. Pollen stainability indicated high pollen fertility.

DISCUSSION

This investigation of the meiotic behaviour of chromosomes and karyotype analysis from mitotic metaphases of Dutch white clover (*T. repens* L.) from Darjeeling Himalaya yielded a number of findings.

Firstly, no meiotic irregularities were observed, and high pollen fertility was determined. Meiotic chromosomes did not form univalents or multivalents in diakinesis and metaphase I. These observations agree with those of Anderson et al. (1991) and Ansari et al. (1999). Atwood and Hill (1940) observed microsporocytes deviating from 16 bivalents at metaphase I. In some of these cells, multivalent pairing was observed. The exceptional cells all showed 15 bivalents and two univalents. In Ladino white clover (*T. repens* L.), Chen and Gibson (1970) had results consistent with those of Atwood and Hill, except that one quadrivalent was observed.

When two genomes in an allopolyploid are divergent, that is, when they have little homology, the pairing of meiotic chromosomes will be regular and only bivalents will be produced at metaphase I. Such an allopolyploid will be fertile, and will be stable both genetically and cytologically. But when two genomes in an allopolyploid are closely related, that is, sufficiently homologous, they will show a variable number of quadrivalents at metaphase I. Such an allopolyploid will be partially fertile, and genetically and cytologically unstable. The formation of regular bivalents and the high pollen fertility observed in the present study demonstrate that allopolyploid *Trifolium repens* is characterised by diploid-like meiotic behaviour of the chromosomes, insuring high fertility of the allopolyploid species. The results also indicate the remoteness of the relationship between its possible progenitors, at least at the genomic level.

Secondly, the mitotic metaphase chromosomes of an allopolyploid should be expected to have at least two pairs of chromosomes having a secondary constriction, or two satellite-bearing chromosomes. However, analysis of the mitotic metaphase of Dutch white clover revealed the karyotype to be symmetrical, and only one pair of chromosome had a satellite. In ladino white clover, Chen and Gibson (1971) observed one pair of chromosomes bearing a satellite. Using FISH technique with 5S and 18S-26S rDNA as probe, Ansari et al. (1999) confirmed the presence of one pair of nucleolus organiser regions on one pair of chromosomes in *Trifolium repens* L. Thus, the mitotic metaphase of Dutch white clover also shows diploid-like somatic chromosome complements. This cytogenetic character of Dutch white clover means favourable genetic and cytological stability in nature. Its high pollen fertility and self-compatibility enhances its usefulness in breeding. Although traditional cytogenetic analysis does not solve the origin or evolution of a species, it can still provide data suggesting the development of genetic mechanisms that cause diploid-like behaviour in chromosomes, mechanisms that may permit the allopolyploid condition to re-enter an essentially diploid-like condition in the course of evolution and during speciation.

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