

Evidence for the existence of endosperm balance number in the true clovers (*Trifolium* spp.)¹

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The endosperm balance number (EBN) hypothesis was first advanced to explain results from interspecific crosses in *Solanum* and later in *Impatiens*. According to the EBN hypothesis, normal endosperm development following intra- or inter-specific crosses depends on having a ratio of two EBNs from the female to one EBN from the male in the endosperm tissue. EBNs may differ among related species. Successful hybrids can be obtained between species with the same EBN. The ploidy level of an individual species can be varied to modify its EBN, making it cross compatible with a species sharing its modified EBN. Inter-specific crosses within *Trifolium* have been limited and difficult. Crosses reported in the literature, including evidence from our own study, suggest that EBN is operating in *Trifolium* and have been used to assign EBN numbers to some clover species. The use of $2n$ eggs enabled two species, differing in EBN, to be crossed. An understanding of the EBN mechanism that operates in *Trifolium* should make successful interspecific hybrids easier to obtain in the future.

Key words: endosperm balance number, hybrids (interspecific), $2n$ gametes, *Trifolium*.

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L'hypothèse de la balance numérique d'un endosperme (BNE) a été formulée pour expliquer les résultats de croisements interspécifiques, d'abord chez *Solanum* et, plus tard, chez *Impatiens*. D'après cette hypothèse, il y a formation d'un endosperme normal lors de croisements intra- ou inter-spécifiques, si le tissu endospermique comporte un ratio de 2 BNE du parent femelle et de une BNE du parent mâle. Les BNE peuvent être différentes chez les espèces reliées. De bons hybrides peuvent être obtenus d'espèces ayant la même BNE. Le niveau de ploïdie d'une espèce particulière peut varier et engendrer une BNE modifiée et cette espèce pourra être croisée avec une autre espèce dont la BNE a été modifiée de la même façon. Les croisements interspécifiques chez *Trifolium* ont été limités et difficiles. Les croisements rapportés dans la littérature, incluant les évidences de la présente étude, suggèrent que la BNE joue un rôle chez *Trifolium* et des nombres BNE (EBN) ont été assignés à certaines espèces de trèfle. Le recours à des oosphères $2n$ a rendu possible le croisement entre deux espèces dont la BNE diffèrait. Une bonne connaissance du mécanisme par lequel la BNE agit chez *Trifolium* devrait permettre l'obtention plus facile d'hybrides interspécifiques ultérieurement.

Mots clés: balance numérique de l'endosperme, hybrides interspécifiques, gamètes $2n$, *Trifolium*.

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Introduction

Advancement of the endosperm balance number (EBN) hypothesis (Johnston et al. 1980) has provided a means to predict successful interspecific crosses in the genus *Solanum* (Johnston and Hanneman 1980, 1982). Arisumi (1982) found evidence that EBN was operational in *Impatiens* and Nishiyama and Yabuno (1979) described the same phenomenon in *Avena*.

According to the EBN hypothesis, each species has an effective ploidy level (termed EBN) that determines its crossing behavior. For a cross (intra- or inter-specific) to be successful, the endosperm must have a ratio of two EBNs from the female parent to one EBN from the male

parent, as normally occurs during intraspecific, intra-ploidy crosses. If the ratio departs from two female to one male EBN, the endosperm breaks down during development, leading to either poor development or death of the embryo (Peloquin et al. 1982).

Barring any stylar or ovular barriers, two closely related species can be expected to cross if they share the same EBN. Two species with unlike EBNs (e.g., EBN = 2 and EBN = 4, respectively) can be crossed by first doubling the chromosome number and consequently the EBN of the first species. The use of a functional $2n$ gamete from the first species is an alternative to doubling its chromosome number.

This paper examines crosses reported in the literature, which suggest that EBN also operates in the true clovers (genus *Trifolium*) and support the designation of EBN

¹Cooperative investigation of USDA-ARS and the Wisconsin Agricultural Experiment Station, Madison, WI.

numbers for some species, indicating that EBN may be a widespread phenomenon. This paper also presents new information on the use of $2n$ eggs to overcome EBN barriers in clover.

Materials and methods

Reports of interspecific hybridization of *Trifolium* in the literature were examined to determine if crossing results could be interpreted under the EBN hypothesis. Further, the use of $2n$ gametes can provide experimental evidence to test if the EBN is functional in clover. If a diploid ($2x$) plant produces both haploid (n) and diploid ($2n$) eggs, the ploidy of the progeny should depend on the EBN of the diploid ($2x$) species used as a pollen parent. If the pollen parent is of the same EBN as the egg parent, the haploid eggs will preferentially survive after fertilization, resulting in diploid ($2x$) hybrid progeny. If the EBN of the diploid pollen parent is twice that of the egg parent, then the embryos formed by the diploid ($2n$) eggs should survive preferentially, resulting in triploid hybrid progeny. This crossing behaviour is diagrammed in Table 1. To test this concept, plants of a diploid red clover, *Trifolium pratense* L. ($2n=2x=14$), Wisconsin breeding line, homozygous for the synaptic (*sy*) mutant gene, were used as female (egg) parents. The *sy* gene causes a failure of the chromosomes to pair during meiosis. The result is male sterility with greatly reduced female fertility (Smith et al. 1985). It also results in the formation of $2n$ eggs in some plants. The frequency of haploid (n) and diploid ($2n$) egg formation in each plant was determined by seed set following pollination with pollen from either diploid or tetraploid red clover plants.

The synaptic-mutant egg parents were then pollinated with pollen of *T. diffusum* ($2n=2x=16$) (P.I. 120144 or P.I. 204517) and *T. pallidum* ($2n=2x=16$) (P.I. 249868). Tetraploid *T. diffusum* ($2n=4x=32$) plants (developed by nitrous oxide chromosome doubling of P.I. 204517, and provided by Dr. N. L. Taylor, University of Kentucky, Lexington, Kentucky, U.S.A.) were also used as a pollen source in crosses with one of the diploid ($2x$) red clover $2n$ egg producing plants. Chromosome numbers of the hybrids were verified in root-tip cells stained with modified carbol fuchsin. Correlations between egg frequency (%) and seed set (no. of seeds/no. of crosses) were calculated following respective pollinations with *T. diffusum* and *T. pallidum*.

Results and discussion

The genus *Trifolium* L. consists of about 240 species, divided into eight sections (Zohary and Heller 1984). A survey of the literature has revealed few hybrids within sections and none between sections. In many cases fertilization takes place, so stylar or ovular barriers can be excluded as reasons for the failure of interspecific crosses (Chen and Gibson 1972). Some of these failures are due to breakdown of the endosperm (Kazimeirski et al. 1972; Williams and White 1976). As EBN ratios other than two female to one male lead to endosperm breakdown, this suggests that EBN might be one of the factors that create interspecific barriers in the *Trifolium*. Enough interspecific hybrids have been obtained to support the establishment of at least a partial EBN series

TABLE 1. Ploidy and EBN of progeny expected from crosses of a $2x$, 2EBN egg parent that produces $2n$ gametes when fertilized with pollen of 4EBN

Egg parent ($2x$, 2EBN)	Pollen parent	
	$2x$, 2EBN	$2x$, 4EBN
n egg	$2x$, 2EBN	None
$2n$ egg	None	$3x$, 4EBN

in two sections of the genus *Trifolium*, namely section *Trifolium* Zoh. and section *Lotoidea* Crantz. A summary of hybrids that have been obtained within the section *Lotoidea*, as well as proposed EBNs to explain the crossing results, are given in Table 2. Similar information is given in Table 3 for section *Trifolium*.

When *T. repens* (white clover), which is a tetraploid, is crossed with *T. occidentale*, a diploid species, the crosses fail (Gibson and Beinhart 1969) (Table 2). This situation is analogous to a cross between a tetraploid and a diploid of the same species. Such crosses commonly fail because of a phenomenon usually known as the "triploid block" brought about by a four female to one male or two female to two male EBN ratio in the endosperm, depending on the direction of the cross. When *T. repens* is crossed with an autotetraploid form of *T. occidentale*, however, the crosses are successful (Gibson and Beinhart 1969). Furthermore, if an octoploid form of *T. repens* is used in crosses with autotetraploid *T. occidentale*, the crosses fail (Gibson and Beinhart 1969) as the "triploid block" (in this case, a "hexaploid" block) is once again operational. The results demonstrate that both *T. repens* and *T. occidentale* can be assigned an EBN that is equal to their ploidy. Using *T. occidentale* as a reference point, its haploid complement (x) would have EBN = 1 and the diploid plant would have EBN = 2. As *T. repens* and *T. occidentale* have an equal EBN per genome, tetraploid *T. repens* has EBN = 4.

Triploid hybrids are easily obtained when $4x$ *T. repens* is crossed with $2x$ *T. nigrescens* (Trimble and Hovin 1960; Hovin 1962). Yet, if $4x$ *T. repens* is crossed with $4x$ *T. nigrescens*, no progeny are obtained (Evans 1962a). When $8x$ *T. repens* is crossed with $4x$ *T. nigrescens*, hexaploid hybrids are obtained (Brewbaker and Keim 1953; Evans 1962a). These results contradict results normally expected if the conventional "triploid block" was operational. Nevertheless, these results are easily explained if *T. nigrescens* is assigned an EBN that is twice as great as its ploidy level, such that $2x$ *T. nigrescens* would have EBN = 4. Successful crosses are those, then, that match equivalent EBNs rather than ploidy levels.

With the EBNs that have been assigned to this point it

TABLE 2. Reported interspecific crosses in the section *Lotoidea* and proposed endosperm balance numbers

Cross		Progeny	Reference
<i>repens</i>	(4x, 4EBN) × <i>occidentale</i> (2x, 2EBN)	— ^a	Gibson and Beinhart 1969
<i>repens</i>	(4x, 4EBN) × <i>occidentale</i> (4x, 4EBN)	4x, 4EBN	Gibson and Beinhart 1969
<i>repens</i>	(8x, 8EBN) × <i>occidentale</i> (4x, 4EBN)	—	Gibson and Beinhart 1969
<i>repens</i>	(4x, 4EBN) × <i>nigrescens</i> (2x, 4EBN)	3x, 4EBN	Trimble and Hovin 1960; Hovin 1962
<i>repens</i>	(4x, 4EBN) × <i>nigrescens</i> (4x, 8EBN)	—	Evans 1962a
<i>repens</i>	(8x, 8EBN) × <i>nigrescens</i> (4x, 8EBN)	6x, 8EBN	Brewbaker and Keim 1953; Evans 1962a
<i>nigrescens</i>	(2x, 4EBN) × <i>occidentale</i> (2x, 2EBN)	—	Chou and Gibson 1968; Gibson and Beinhart 1969
<i>nigrescens</i>	(2x, 4EBN) × <i>occidentale</i> (4x, 4EBN)	3x, 4EBN	Chou and Gibson 1968; Gibson and Beinhart 1969
<i>nigrescens</i>	(4x, 8EBN) × <i>occidentale</i> (4x, 4EBN)	—	Gibson and Beinhart 1969

^aUnsuccessful cross.TABLE 3. Reported interspecific crosses in the section *Trifolium* and proposed endosperm balance numbers

Cross		Progeny	Reference
Subsection <i>Alpestris</i>			
<i>sarosiense</i>	(6x, 8EBN) × <i>alpestre</i> (2x, 4EBN)	— ^a	Quesenberry and Taylor 1978
<i>sarosiense</i>	(6x, 8EBN) × <i>alpestre</i> (4x, 8EBN)	5x, 8EBN	Quesenberry and Taylor 1978; Maizonnier 1972 ^b
<i>alpestre</i>	(2x, 4EBN) × <i>heldreichianum</i> (2x, 4EBN)	2x, 4EBN	Quesenberry and Taylor 1976
<i>alpestre</i>	(2x, 4EBN) × <i>rubens</i> (2x, 4EBN)	2x, 4EBN	Quesenberry and Taylor 1976
Subsection <i>Trifolium</i>			
<i>pratense</i>	(2x, 3EBN) × <i>pallidum</i> (2x, 6EBN)	—	Armstrong 1968; Armstrong and Cleveland 1970
<i>pratense</i>	(4x, 6EBN) × <i>pallidum</i> (2x, 6EBN)	3x, 6EBN	Armstrong and Cleveland 1970; Schwer and Cleveland 1972b
<i>pratense</i>	(2x, 3EBN) × <i>diffusum</i> (2x, 3EBN)	2x, 3EBN	Taylor et al. 1959, 1963; Schwer and Cleveland 1972a; Rubtsov and Komkova 1983
<i>pratense</i>	(4x, 6EBN) × <i>diffusum</i> (4x, 6EBN)	4x, 6EBN	Taylor et al. 1963; Rubtsov and Komkova 1983
<i>pratense</i>	(4x, 6EBN) × <i>diffusum</i> (2x, 3EBN)	—	Schwer and Cleveland 1972b
Subsection <i>Alpestris</i> × subsection <i>Trifolium</i>			
<i>sarosiense</i>	(6x, 8EBN) × <i>alpestre</i> (2x, 4EBN)	4x, 6EBN ^c	(Maizonnier 1972)
<i>sarosiense</i>	× <i>alpestre</i> (4x, 6EBN)	—	—
	× <i>pratense</i> (4x, 6EBN)	(4x, 6EBN) ^d	(Maizonnier 1972)

^aUnsuccessful cross.^bUsed 2n pollen grain from 2x *T. alpestre*.^cRare hybrid (3 hybrids from 1750 crosses).^dAlbino seedlings.

is possible to predict that crosses between *T. nigrescens* and *T. occidentale* at the same ploidy level would fail, as they would have unequal EBNs. Evidence from the literature supports these conclusions (Chou and Gibson 1968; Gibson and Beinhart 1969) (Table 2). The use of 4x *T. occidentale* is necessary to equate its EBN with that of 2x *T. nigrescens*. Such crosses would be expected to be successful. This is, in fact, the case (Chou and Gibson 1968; Gibson and Beinhart 1969).

The same type of reasoning can be applied to interspecific crosses in the section *Trifolium* (Table 3). Hexaploid *T. sarosiense* will not cross with 2x *T. alpestre* (Quesenberry and Taylor 1978) but will cross with 4x *T. alpestre* (Quesenberry and Taylor 1978;

Maizonnier 1972). Therefore, 6x *T. sarosiense* and 4x *T. alpestre* share the same EBN (EBN = 8) despite their differences in ploidy level.

It should be emphasized at this point that the use of 2n gamete (i.e., numerically unreduced) from a 2x plant is equivalent to the use of an n gamete from a 4x plant, as both such gametes would have the diploid (2x) complement of chromosomes. Therefore, 2n gametes provide an alternative to chemical tetraploidization of diploid plants to overcome EBN barriers. Conversely, if crosses between species with unlike EBNs are attempted, EBN will act as selective screen for 2n gametes. This was what may have happened when Maizonnier (1972) crossed 6x *T. sarosiense* with 2x *T. alpestre* (Table 3).

TABLE 4. Frequency of haploid (n) and diploid ($2n$) eggs produced by 17 synaptic-mutant *T. pratense* plants and percent seed set following intra- and inter-specific pollinations

Egg parent ^a	Pollen parent											
	<i>2x T. pratense</i>			<i>2x T. diffusum</i>			<i>4x T. pratense</i>			<i>2x T. pallidum</i>		
	No. of crosses	No. of seeds	% n eggs	No. of crosses	No. of seeds	% seed set	No. of crosses	No. of seeds	% $2n$ eggs	No. of crosses	No. of seeds	% seed set
1	60	9	15.0	84	3	3.6	69	5	7.3	147	2	1.4
2	122	20	16.4	120	4	3.3	160	5	3.1	261	0	0.0
3	62	5	8.1	174	6	3.4	90	2	2.2	272	0	0.0
4	88	13	14.8	120	5	4.2	84	4	4.8	155	0	0.0
5	75	12	16.0	151	14	9.3	94	0	0.0	134	0	0.0
6	96	8	8.3	185	2	1.1	79	0	0.0	205	0	0.0
7	104	7	6.7	135	0	0.0	108	0	0.0	—	—	—
8	63	59	93.7	93	29	31.2	110	4	3.6	—	—	—
9	64	4	6.3	64	4	6.3	153	5	3.3	33	0	0.0
10	83	21	25.3	126	2	1.6	70	7	10.0	262	4	1.5
11	61	11	18.0	120	12	10.0	75	4	5.3	169	3	1.8
12	54	14	25.9	127	25	19.7	71	0	0.0	136	1	0.7
13	83	68	81.9	111	12	10.8	60	0	0.0	122	0	0.0
14	69	3	4.3	80	0	0.0	72	0	0.0	—	—	—
15	77	63	81.8	110	24	21.8	80	0	0.0	108	0	0.0
16	69	12	17.4	111	6	5.4	93	2	5.4	140	2	1.4
17	180	6	3.3	4	1	2.5	180	13	2.5	50	3	6.0
Total	1410	335	23.8	1951	149	7.6	1648	51	3.1	2194	15	0.7

^aDiploid ($2x$) *T. pratense*.

All these crossing results suggest that EBN is functioning and that diploid ($2x$) *T. alpestre* shares the same EBN with $2x$ *T. heldreichianum* and $2x$ *T. rubens* (Quesenberry and Taylor 1976) (Table 3). Tetraploid *T. pratense* (red clover) shares the same EBN with $4x$ *T. diffusum* and $2x$ *T. pallidum* (Taylor et al. 1959; Taylor et al. 1963; Armstrong 1968; Armstrong and Cleveland 1970; Schwer and Cleveland 1972a, 1972b; Rubtsov and Komkova 1983). Furthermore, Armstrong (1968) has documented the breakdown of endosperm tissue in crosses of $2x$ *T. pratense* \times $2x$ *T. pallidum*. This is expected to occur under the EBN hypothesis, as these species have unequal EBNs at the same ploidy level.

Within section *Trifolium*, subsection *Alpestris* (*T. alpestre*, *T. sarosiense*, *T. heldreichianum*, and *T. rubens*) forms a group with a degree of interspecific cross compatibility. Subsection *Trifolium* (*T. pratense*, *T. diffusum*, and *T. pallidum*) forms another group with interspecific cross compatibility within the same section. The exact EBN relationship between these subsections is still unclear. A clue was provided by Maizonnier (1972), who was able to obtain 3 interspecific, inter-EBN hybrids from a total of 1750 crosses (Table 3). These hybrids could then be crossed with $4x$ *T. pratense*, indicating that they probably share a common

EBN. This information was then used to relate the EBNs between subsection *Alpestris* and subsection *Trifolium*.

It has been emphasized (Johnston et al. 1980; Johnston and Hanneman 1980) that matching EBN is a necessary but not sufficient condition for a successful cross. Successful crosses in species with the same EBN may be prevented by prefertilization barriers unrelated to EBN. Such barriers are well known in *Trifolium*. The failure of pollen tubes to grow after interspecific crosses has been well documented by Muller (1960), Evans (1962b), and Kazimierska (1978a). In addition, crosses between species differing in EBN can be successful, albeit at a very low rate. In this paper, unsuccessful crosses, or those with a very low success rate (e.g., 1% or less), have been taken to indicate a difference in EBN if prefertilization barriers can be reasonably excluded as a cause of incompatibility. Successful crosses (those with a success rate of about 10% or greater) have been taken as an indication of like EBN.

The results of crosses between $2x$ and $4x$ *T. pratense* are given in Table 4. Between 3.3 and 93.7% of the flowers in the synaptic-mutant egg parents produced haploid (n) eggs with an average frequency of 23.8% n eggs as demonstrated in crosses with $2x$ *T. pratense*. Zero to ten percent of the flowers in the same plants produced $2n$ (diploid) eggs with an average frequency of

3.1% $2n$ eggs as indicated by crosses with $4x$ *T. pratense*.

When *T. diffusum* was used as a pollen parent, seed set (7.6%) and the frequency of haploid eggs produced by the synaptic-mutant *T. pratense* parents (23.8%) were highly correlated ($r = 0.81$; $p < 0.01$). Seed set (0.7%) and the frequency of $2n$ eggs (3.1%) were also correlated ($r = 0.60$; $p < 0.05$) when *T. pallidum* was used as a pollen parent. Chromosome counts of the progeny revealed the expected $2n=2x=15$ chromosomes for the *T. pratense* \times *T. diffusum* progeny. The *T. pratense* \times *T. pallidum* hybrids had the expected $2n=3x=22$ chromosomes. It was also expected that embryos which developed from $2n$ eggs would survive preferentially in the $2x$ *T. pratense* \times $4x$ *T. diffusum* crosses to give $4x$ hybrid progeny. Nine progeny were obtained from 421 crosses. The ploidy of two of these hybrids was examined and found to be as expected. Although eggs of two different ploidies were present and probably fertilized, embryo survival depended on the species used as a pollen parent. It is this difference in embryo survival that suggests an EBN relationship is operating among *T. diffusum*, *T. pallidum*, and *T. pratense*.

The EBN numbers that have been proposed are valid only within each section. The exact EBN relationships between sections are unknown at present, and their elucidation must await successful crosses between sections. There is evidence for EBN barriers between sections in addition to those within sections. For example, Kazimierska (1978b) has reported that crosses between *T. repens* L. and *T. medium* L. fail because of a breakdown of the endosperm. When successful crosses can be made between sections, the proposed EBNs for one section or the other may have to be modified to accommodate the new information.

The endosperm balance number concept helps explain why *T. pratense* has been so reproductively isolated from related perennial species and lays a base for further understanding of crossability barriers within each section. It is hoped that a better understanding of EBN relationships within *Trifolium* will challenge breeders to identify potentially successful interspecific crosses and provide the opportunity to manipulate EBN through the use of $2n$ gametes and (or) colchicine treatment to obtain interspecific hybrids.

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