Appendix
CHIASMATE ASSOCIATION IN DIPLOID AND THE AUTOTETRAPLOID OF MEDICINAL PLANT ANDROGRAPHIS PANICULATA, AND ITS BEARING ON SEED FERTILITY

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Andrographis paniculata Nees (fam. Acanthaceae) occurring wild throughout Indian subcontinent is highly valued for its diverse medicinal uses as a bitter, liver toner, and as antispasmodic, antiperistaltic, stomachic, etc. (10). Its biomass is the chief source of the crude drug as well as valuable secondary metabolites. Data reported in the literature suggest that artificial polyploidy could be a rapid means to attain enhanced production of secondary metabolites in such situations, both by way of increasing the biomass as well as the concentration of secondary metabolites (1). Also, elevated ploidy level may bring about qualitative changes in the metabolic profile on account of differential gene expression (2,4). Of course, these effects are not always observed and may be species specific (3), but are sufficient to be searched for and exploited in crop breeding.

However, incidence of low seed set generally encountered in the induced polyploids imposes serious problem in the realisation of polyploid stability, even if the seed is not the source of economic product, such as in Andrographis paniculata. Therefore, in order to elucidate the prospects of polyploidy breeding in this species, a quantitative analysis of meiotic associations vis-a-vis possible genetic stability in the progenitor diploids and the derived autopolyploids over two generations was undertaken, the results of which are reported here.

Material and methods

Homogenous seed material obtained by self pollination from a local population of Andrographis paniculata (2n = 50) was used for the induction of polyploidy. Shoot tips of fast growing seedlings at 4-6 leaf stage were treated with 0.2% aqueous solution of colchicine (Sigma) with a cotton swab for 24 hours. On the basis of deep green coloration, thick texture and deformed morphology of first leaves appearing after treatment, 10 plants were selected as potential tetraploids. These were subsequently transferred to field for optimal cultural care and scored for enlarged size of stomatal guard cells in the leaves. Majority of plants exhibited mixture of small and large stomata, and only three had larger stomata present uniformly. The latter three raw autotetraploids (termed as C0) were allowed to grow further, and their seeds were collected to raise C1 progenies.

Although, this plant is propagated through seeds, but with proper care it could be made possible to propagate it vegetatively by layering. Therefore, to have realistic comparison, all the three plant types i.e. vegetative progenies of diploid progenitor, C0 raw tetraploids and C1 seed progenies were grown simultaneously to...
collect data on meiotic behaviour and seed fertility. For meiotic analysis, flower buds of appropriate size were collected between 800 to 900 hrs. from plants grown in the field, and were fixed overnight in Carnoy's (6:3:1) fixative, and again transferred to fresh lot of the fixative for another 24 hrs. At least 10 well analyzable cells from three flower buds each at metaphase I, from diploid, C₀ and C₁ generations were scored for meiotic chromosome associations, using acetocarmine squash technique. Seed set in all the plants was scored on the basis of healthy (fertile) vs shrivelled (sterile/ nonviable) seeds, healthy seeds taken to estimate % seed set.

Observations and deductions

For a uniform comparison of meiotic associations in the progenitor diploids and their derived autotetraploids, the data recorded for given type of configurations have been transformed in terms of frequency. They are expressed as a set of four for tetraploids and two for diploids; x = 25 in Andrographis paniculata. The data for meiotic configurations, estimations on chiasmate association, and seed set are shown in table 1.

Sybenga (12) has provided an algebraic approach to estimate multivalent pairing (t), and chiasmate association of the average long arm 'a' and short arm 'b'. A more simplified version is given in Lavania (5,6). Following algebraic equations were used for the purpose.

(1) For tetraploids:

multivalent pairing (t) = (t+2cq+4rq)² / 16rq

a.b = rq + 1/2 cq + 1/4 t + r

a + b - 2ab = 1/2 cq + 1/4 t + q

The symbols t, rq, cq, r, o, u represent the frequency of trivalents + accompanying univalents, ring quadrivalents, chain quadrivalents, ring bivalents, open bivalents, and independent univalent pairs; a and b represent weighted average of chiasmate (bound arm) association of average long and average short arm, respectively.

(2) For diploids:

frequency of ring bivalents (r) = a.b

frequency of open bivalents (o) = a+b - 2ab

Further, the individual values of 'a' and 'b' in all cases could be deduced using the quadratic equation:

\[ a, b \text{ respectively } = \frac{(a+b) \pm \sqrt{(a+b)^2 - 4ab}}{2} \]

Discussion

Artificial autopolyploidy is usually unattractive for seed propagated crops because of reduced seed set and seed inviability on account of meiotic irregularities and disturbance of reproductive physiology. Even in crops where not the seed itself but plant biomass constitutes the economic product such as in Andrographis paniculata, seeds are still required for propagation. In addition, high seed set is often accompanied by genetic stability, as both depend, at least in part, on the same mechanisms (7). Data presented here show that artificial polyploidy did not lead to significant meiotic disturbances, nor to seed set in Andrographis paniculata.

Reasonably good seed fertility in artificial autotetraploids i.e. over 65% has earlier been recorded in several species that have small chromosomes and distal chiasma localisation. In all such systems, the high seed set was observed to be on account of higher than expected bivalent pairing in the autopolyploids (further details in 6). The estimations derived from the data given in table 1 reveal that melosis in Andrographis...
Table 1: Frequency of meiotic configurations, chiasmate (bound arm) association, and seed fertility in the progenitor diploid and its autotetraploid in *Andrographis paniculata*

<table>
<thead>
<tr>
<th>Type of Chromosome associations</th>
<th>Diploid</th>
<th>Autotetraploid</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rq</td>
<td>0.188</td>
<td>0.172</td>
</tr>
<tr>
<td>cq</td>
<td>0.288</td>
<td>0.248</td>
</tr>
<tr>
<td>t</td>
<td>0.080</td>
<td>0.076</td>
</tr>
<tr>
<td>r</td>
<td>0.392</td>
<td>0.066</td>
</tr>
<tr>
<td>q</td>
<td>0.60</td>
<td>0.378</td>
</tr>
<tr>
<td>u</td>
<td>0.008</td>
<td>0.020</td>
</tr>
<tr>
<td>f</td>
<td>62.21</td>
<td>57.69</td>
</tr>
<tr>
<td>a</td>
<td>0.986</td>
<td>0.889</td>
</tr>
<tr>
<td>b</td>
<td>0.398</td>
<td>0.459</td>
</tr>
<tr>
<td>a+b</td>
<td>1.384</td>
<td>1.348</td>
</tr>
<tr>
<td>Seed set (%)</td>
<td>64</td>
<td>41</td>
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<td></td>
<td>44</td>
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*Andrographis paniculata* at tetraploid level entails higher bivalentisation and low quadrivalent pairing (f = 62.2% in C<sub>0</sub> compared to 66.7% expected on random pairing) that undergoes further reduction in subsequent C<sub>1</sub> generation. The relatively low levels of chiasmate association and the clear difference in chiasma formation between the average two arms per chromosome should have resulted in lower observed than expected multivalent association frequency. Such differences in the chiasmate association frequency of the two chromosome arms are further enlarged in the subsequent autotetraploid generation C<sub>1</sub>, thus facilitating further decrease in quadrivalent and increase in bivalent frequency, and in turn more regular meiotic segregation and improved seed set.

From a closer look at the observed meiotic data in the diploid and the derived autotetraploids, it appears that the interference phenomenon, combined with distal localisation of the point of partner exchange, either from the start of synaptonemal complex formation or resulting from centrifugal shift or both, may be causing lower multivalent pairing in the autopolyploids (8).

It has earlier been reported that tremendous variation in karyomorphological diversity is present in *Andrographis paniculata* occurring in the Indian subcontinent (9). It is expected that such biotypes may also differ in chiasmate association characteristics. As such, genotypic selection of diploid progenitors for lower chiasmate association should facilitate further improvement in their autotetraploids, as proposed (6).

**Summary**

The artificial autotetraploids developed in the medicinal plant *Andrographis paniculata* (2n = 50) evince reasonably good seed set (relative seed set of over 80%) suitable for utilization in a polyploid breeding programme. Meiotic examination of progenitor diploids vis-a-vis derived autotetraploids examined over two consecutive generations reveal that multivalent association frequencies at metaphase I in the
tetraploids (f = 0.62 in C₀ and 0.57 in C₁) were lower than expected (f = 0.67). This emanates from reduction in chiasmate association of the two chromosome arms when changed to tetraploid state, as also on account of enhanced differences in the association frequency of the two average chromosome arms in the subsequent generation. The data supports the contention that progenitor diploids with high frequency of open bivalents/low chiasma frequency should enhance the fertility of the artificial polyploids.

References


Bacopa monnieri (L) Pennell, a prostrate herb, 2n=64, family Scrophulariaceae occurs world wide in the warmer regions along river banks, ditches and water bodies. The entire plant is the source of active principles, and the biomass as such constitutes an important commercial drug of the Indian Materia Medica called "Brahmi", commonly used in the treatment of mental disorders, asthma, and as a cardiotonic. Plant extract has relaxant (2), antioxidant (8,11), anticancer (4,14) and analgesic (15) properties. Standardized extracts of the plant rich in active constituents bacosides, mainly bacoside A have recently been developed as a clinical drug for memory and intellect improvement (1,9,10).

Since plant biomass and its constituent active principles are the source of the Brahmi drug and drug derivatives, there exists a possibility to realize rapid genetic improvement of Bacopa monnieri through polyploid intervention. This seems to be so because artificial polyploidy is known to facilitate quantitative increase in biomass and quantitative and qualitative changes in secondary metabolites (3,5). Further, inspite of predominant vegetative mode of reproduction, this species also harbours seed formation (7). Latter offers unique opportunity to realize desirable genomic combinations, through sexual recombination. In addition to autotetraploid, triploid heterosis could be harnessed upon and easily sustained through vegetative propagation. However, as a first step it is necessary to understand chromosome behaviour in the diploids and its bearing on tetraploid meiosis and gametic fertility to elucidate the prospects of triploid formation. A quantitative account of attendant meiotic properties is provided in this communication to add value to autotetraploid perse as well as autotetraploid mediated triploid programme.

Material and methods
Vegetative progeny of a single plant of Bacopa monnieri collected from Lucknow was used as the source material. Fast growing shoot-tips of transplanted shoot at 10-12 leaf stage were treated with 0.2% aqueous solution of colchicine (Sigma) by cotton swab method for 36 hrs for polyploid induction. Treated plants were screened on the basis of deformed morphology, deep green coloration, thick texture and broad size of the leaves. Ten such plants were scored for enlarged size of stomatal guard cells, and further screened for stomatal uniformity over the vegetative growth period. Vegetative progenies were grown for cytological analysis.

For mitotic studies, healthy root tips (1-2 cm) were excised and pretreated in a saturated aqueous solution of para-dichlorobenzene for 3 hrs at 12-14°C, fixed overnight in Carnoy's fixative (6:3:1), and mitotic squashes prepared following hydrolysis in 1N.HCl for 5 min at 60°C.
followed by staining in 2% aceto-carmine. For meiotic analysis floral buds excised between 0800 and 0900 hrs were fixed in Carnoy's fixative (6:3:1) for 24hrs, and PMC squashes prepared in 2% aceto-carmine. At least 10 well analysable cells from three flower buds each at metaphase I and anaphase I from progenitor diploid and the derived autotetraploid were scored for meiotic chromosome associations and anaphase segregation. Pollen fertility was scored by aceticarmine staining method.

Observations and deductions
For a uniform comparison of meiotic associations in the progenitor diploids and their derived autotetraploids, the data recorded for given type of configurations have been transformed in terms of frequency. They are expressed as a set of four for tetraploids and two for diploids; x=32 in *Bacopa monnieri*. The data for meiotic configurations, estimations on chiasmate association, anaphase I segregation, and pollen fertility are shown in table 1, and representative cytological features are shown in figure 1.

Sybenga (12) has provided an algebraic approach to estimate multivalent pairing (f), bivalent pairing (1-f), and also the chiasmate association of the average long arm (a) and short arm (b). A more simplified version is given in Lavania (6), and further simplified here for a ready reference. Following algebraic equations were used for the purpose:

**(1) for tetraploids:**

multivalent pairing (f) = \((t+2cq+4rq)^2 / 16rq\)

\[ a.b = rq + \frac{1}{2} cq + \frac{1}{4} t + r \]

\[ a+b - 2ab = \frac{1}{2} cq + \frac{1}{4} t + o \]

The symbols t, rq, cq, r, o, u represent the frequency of trivalents + accompanying univalents, ring quadrivalents, chain quadrivalents, ring bivalents, open bivalents and independent univalent pairs; a and b represent weighted average of chiasmate (bound arm) association of average long and average short arm, respectively.

**(2) For diploids:**

frequency of ring bivalents (r) = a.b

frequency of open bivalents (o) = a+b - 2ab

Further, the individual values of 'a' and 'b' in all cases could be deduced using the quadratic equation:

\[ a, b \text{ respectively} = \frac{(a+b) \pm \sqrt{(a+b)^2 - 4ab}}{2} \]

Discussion
Although, any genetic or genomic change once introduced in *Bacopa monnieri* could...
be easily sustained on account of its predominant vegetative mode of reproduction, but to further utilize the introduced change or inherent potential through recombination breeding and meiotic sieve, it would of value if the pattern of meiotic behaviour is made available in order to plan further experimentation on genomic manipulation and predict the envisaged change. Knowledge of meiotic association and anaphase segregation in the autotetraploid could provide clues to the success and extent of stability of triploid breeding programme in particular and polyploid breeding in general.

Presence of four homologous chromosomes in autotetraploids will naturally favour the formation of bivalents or quadrivalents, both of which in theory should disjoin regularly at anaphase I of meiosis. However, the occurrence of trivalents and univalents, or the linear orientation of quadrivalents with centromeres amphitelic, causes a considerable risk of unbalanced segregation (6, 13). In the present study, the observed multivalent association f = 0.636 in the autotetraploids is almost on expected lines of 2/3 (i.e. 0.667), but there is considerable amount of unbalanced anaphase segregation leading to nearly 40% reduction in gametic (pollen) fertility in the autotetraploids. Whatever, small reduction for multivalent association is there i.e. 0.031, it is mainly on account of open bivalents in the progenitor diploids. This is clearly reflected from the bound arm association of the two arms in the diploids and the autotetraploids. Obviously, higher incidence

<table>
<thead>
<tr>
<th>Type of chromosome associations</th>
<th>Diploid</th>
<th>Autotetraploid</th>
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<tbody>
<tr>
<td></td>
<td>Observed no ± SD</td>
<td>Observed no ± SD</td>
</tr>
<tr>
<td></td>
<td>(estimated frequency)</td>
<td>(estimated frequency)</td>
</tr>
<tr>
<td>ring quadrivalents / rq</td>
<td>11.17 ± 2.12 (0.349)</td>
<td>26.875 ± 1.54 (0.840)</td>
</tr>
<tr>
<td>chain quadrivalents / cq</td>
<td>6.67 ± 1.49 (0.206)</td>
<td>4.75 ± 1.39 (0.146)</td>
</tr>
<tr>
<td>trivalents + accompanying l / t</td>
<td>2.33 ± 1.79 (0.073)</td>
<td>0.375 ± 0.48 (0.012)</td>
</tr>
<tr>
<td>ring bivalents / r</td>
<td>26.875 ± 1.54 (0.840)</td>
<td>14.83 ± 3.16 (0.232)</td>
</tr>
<tr>
<td>open bivalents / o</td>
<td>4.75 ± 1.39 (0.146)</td>
<td>8.33 ± 2.43 (0.130)</td>
</tr>
<tr>
<td>univalent pairs / u</td>
<td>0.375 ± 0.48 (0.012)</td>
<td>0.5 ± 0.76 (0.006)</td>
</tr>
<tr>
<td>multivalent pairing / f</td>
<td>0.636</td>
<td></td>
</tr>
<tr>
<td>bound arm association of long arm / a</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>bound arm association of short arm / b</td>
<td>0.828</td>
<td>0.658</td>
</tr>
<tr>
<td>chiasmate association of two arms / a+b</td>
<td>1.828</td>
<td>1.658</td>
</tr>
<tr>
<td>Anaphase I segregation (%)</td>
<td>92.2 33.4</td>
<td></td>
</tr>
<tr>
<td>Equal / balanced</td>
<td>92.2</td>
<td>33.4</td>
</tr>
<tr>
<td>Unequal by : 2- 4 I</td>
<td>6.8</td>
<td>24.0</td>
</tr>
<tr>
<td>Unequal by : &gt; 4 I</td>
<td>–</td>
<td>42.6</td>
</tr>
<tr>
<td>Pollen fertility</td>
<td>90.4</td>
<td>53.0</td>
</tr>
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of open bivalents in the progenitor diploids should impart further reduction in multivalent association in the derived autotetraploids, and consequently an improved balanced anaphase I segregation and gametic fertility. As such, the observations and expectations are in accordance to assumption to the genotypic strategy proposed by Lavania (6), and call for the efforts to look for progenitor diploids with higher occurrence of open bivalents in order to realize higher gametic fertility in the autotetraploids. This would facilitate development of genetically balanced triploids in Bacopa monnieri.

Summary

The artificial autotetraploids of Bacopa monnieri, 2n = 64, 4x = 128, evince cytological stability through somatic and meiotic sieves. The observed multivalent association in the autotetraploids is on expected lines exhibiting multivalent pairing frequency of 0.636, and there is only a small reduction in bound arm association frequency from 1.828 in the progenitor diploids to 1.658 in the derived, autotetraploids. However, considerable disturbances occur in anaphase I segregation leading to reduction in gametic fertility by 40%. Analysis of attendant meiotic configurations suggests that selection for progenitor diploids with higher incidence of open bivalents may impart more balanced anaphase segregation and improved gametic fertility.

References