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Tissue Culture in *Haworthia*

Part 4: Genetic Characterization of Plants Regenerated from Callus*

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Summary. Plants regenerated on two different media (NK and I) from the calluses of simple or cloned subcultures, which were originated from a single stock callus of *Haworthia setata* derived from its flower bud, were observed for eight characters, i.e., somatic chromosome number in root tips, growth vigor, leaf shape, leaf color, number of stomata per unit leaf area, esterase zymogram, chromosome association at meiotic metaphase I in pollen mother cells, and pollen fertility. From these regenerates plants with different characters from those of the parental plant were obtained. With regards to chromosomal aberrations, tetraploids, aneuploids, plants with a part of the chromosome segment deleted, with reciprocal and non-reciprocal translocations, or with paracentric inversions and those showing sub-chromatid aberrations at meiosis were obtained. The NK medium tended to regenerate more tetraploids and less plants carrying translocation than the I medium.

Chromosome variabilities in somatic cells of the regenerates correlated with those of the calluses, from which they regenerated, while they did not correlate with either the meiotic irregularities (chromosome association at MI) or pollen fertility of the regenerates. From these facts, it was concluded that a rather large number of callus cells participate in the regeneration of an individual plant, although, however, only a few limited types of the cells form its germ line.

Polyploidy affected growth vigor, leaf shape, stomata number and chromosome association at MI, but its effects were not detected on other characters. Chromosomal aberrations at the diploid level produced no clear changes in the regenerate's phenotype except in meiotic chromosome configuration and pollen fertility.

Most chromosomal variants obtained in the present study are already reported in plants collected from wild populations, but plants with the deletion of a whole chromosome (karyotype 7L + 6S) or chromosome segment (7L + 1M + 6S and 14L + 2M + 12S) have never been reported: this fact suggests that tissue culture is a powerful tool for producing plants with novel karyotypes.

Key words: Tissue culture – Plant regeneration – Chromosomal variants – Cytogenetics – *Haworthia*

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Introduction

Many variant plants have been regenerated by tissue culture: by gene mutation (Maliga et al. 1979; Widholm 1974; Sung et al. 1974; Chaleff and Carlson 1974; Gengenbach et al. 1977 and others), chromosome rearrangement (Sacristán and Wendt-Gallitelli 1971; Maliga et al. 1979), genome multiplicity (Nitsch and Nitsch 1969; Nishi and Mitsuoka 1969, Sunderland et al. 1974 and many others), or epigenetic alteration (Turgeon et al. 1976; Meins and Binns 1977). In addition, variability in chromosome number is a well known occurence in regenerates (Heinz and Mee 1971; Ogura 1976) as well as in cells of cultured tissue (D'Amato 1977; Sunderland 1977). In certain cases, regenerated plants were found to be mixoploid (Bennici and D'Amato 1978; Bennici 1979). Recently, Shepard et al. (1980) reported that many variant plants with chromosomal disturbances, such as growth habit, maturity date, tuber characteristics and disease resistance, as well as those with normal karyotypes, could be obtained from protoplast in potato. Such variability of chromosome constitutions in cultured tissue and regenerated plants has hindered (to some extent) the use of tissue culture in genetic and biochemical studies (King et al. 1978). On the other hand, the use of tissue culture could lead to production of novel genotypes or chromosomal complements useful in basic study and plant breeding (Sunderland 1977).

Plants of the genus *Haworthia* that belong to the tribe *Aloineae* of *Liliaceae* have bimodal chromosomes, namely, large (L) and small (S) chromosomes (2n = 14; 8L + 6S, Sato 1942). The large chromosome (L_1 to L_4) can be identified by their respective homologues. On the contrary, identification of the small individual chromo-

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somes is impossible. This karyotype is widespread throughout the *Aloineae*, and has consistent stability. Strong selection seems to be operating for the maintenance of this basic karyotype. However, some anomalies (e.g., interchanges, deletions, duplications and inversions) can be in natural populations and they occasionally occupy a certain niche (Brandham 1974; 1976; Brandham and Johnson 1977), probably because *Haworthia* plants are outbreeding and can be propagated vegetatively. Interchange homozygotes have never been discovered (Brandham 1976; Riley and Majunder 1968).

The callus of *Haworthia setata*, which is a eudiploid, typical bimodal karyotype, shows responses to three auxins and kinetin on its greening and organ redifferentiation that are different from that of tobacco (Ogihara 1979). Many regenerated plants can be selected from calluses cultured under different conditions (Ogihara and Tsunewaki 1979). In the present investigation, somatic chromosome number, growth vigor, leaf shape, leaf color, number of stomata per unit leaf area, esterase zymogram, chromosome association at metaphase I in pollen meiosis, and pollen fertility of the regenerates are studied in order to clarify the variation occurring among the regenerates. In addition, the relationship between the mode of the variation and the culture condition of the calluses are analyzed. Finally, karyotypic changes found among the regenerates are compared with those in the variant karyotypes found in nature.

Materials and Methods

Callus Cultures

Four different callus cultures were used in the present investigation (Table 1). All of them were originally derived from a single stock callus of flower bud origin. Two different methods were adopted to maintain the callus cultures; one was a simple subculture method where a single piece of the parental callus was inoculated onto fresh medium at each subculture. The other was cloning where a parental callus was divided into several pieces, which were all used as inoculum for the next culture generation.

Table 1. No. of regenerated plants from various calluses

Culture No.	Methods of subculture	Preculture light condition	Redifferent. medium[a]	No. examined plants
1	simple	light	NK	81
2	simple	dark	I	60
3	simple	light	I	100
4	cloning	dark	I	147
Total				388

[a] The NK medium was RM-1964 basal medium supplemented with 5 mg/l NAA and 0.1 mg/l kinetin, and the I medium was the same basal medium with 0.1 mg/l IAA added

Culture Conditions

Before transferring each callus to a regeneration medium some of the callus cultures (Culture Nos. 1, 3) were placed under continuous fluorescent illumination (ca. 1000 lux) for two subculture generations (about three months), while the others (Culture Nos. 2, 4) were kept in the dark (Table 1).

Two regeneration media were used to restore plantlets from the calluses (Table 1); (1) NK medium – RM-1964 basal medium (Linsmaier and Skoog 1965) supplemented with 5 mg/l NAA + 0.1 mg/l kinetin, and (2) I medium – RM-1964 basal medium added with 0.1 mg/l IAA (Ogihara 1979). During plant regeneration, all calluses were placed under continuous fluorescent illumination.

Observations

Plantlets redifferentiated from the calluses were transplanted into pots filled with sterilized soil, and were grown for two or more years in an air-conditioned greenhouse (25 C during the day and 18 C at night). The number of plants that attained maturity is shown in the last column in Table 1. Observations were made on eight characters as follows:

(i) Somatic chromosome number: Root tips were pretreated with 0.002 M 8-hydroxyquinoline for 3 hr at room temperature and then fixed with 1 : 3 acetic alcohol. Cytological preparations were made according to the ordinary acetocarmine squash method.

(ii) Growth vigor: Plants were classified into the following three classes for convenience' sake depending upon their growth vigor; 1: poor growth, 2: fair growth, and 3: vigorous growth.

(iii) Leaf shape: Using two randomly selected leaves, their length, width and thickness were measured. Two indices, leaf length/leaf width and leaf length/leaf thickness were calculated to show leaf shape.

(iv) Leaf color: Plants were visually classified into the following five classes; normal green, pale green, pale yellow, red green, and red.

(v) Number of stomata per unit leaf area: Lower surface of the middle part of a fully developed leaf per plant was impressed on a cellulose film by the SUMP method (Kihara and Katayama 1960). The impression of each leaf was photographed and the number of stomata per 0.25 mm^2 leaf area was counted, from its print.

(vi) Esterase zymogram: Two mature leaves were collected per plant. About 200 mg of these leaves was homogenized in 1 ml of potassium phosphate buffer (0.05 M, pH 7.0). The homogenate was centrifuged at 15000 × g for 15 min at 0 C, and the supernatant was used as the crude enzyme extract. Polyacrylamide gel (7.5% in concentration) containing the carrier ampholite with a pH range of 3.0 to 10.0 in a final concentration of 1% was cast in 80 mm long glass tubes with an inside diameter of 5 mm. A 0.2 ml portion of the crude enzyme extract was placed on the top of the gel. Electrophoresis and gel staining were performed according to the method of Nakai and Tsunewaki (1971). The substrate and dye used were α-naphtyl acetate and Fast Blue RR salt, respectively.

(vii): Chromosome association at meiotic metaphase I in pollen mother cells (PMCs).

Flowering buds containing PMCs at metaphase I were collected in March and April, 1979, fixed with 1 : 3 acetic alcohol, and squashed in 1% acetocarmine.

(viii) Pollen fertility: Pollen grains were excised from the anthers of newly flowered buds, and stained with 1% acetocarmine. Morphologically abnormal pollen grains were judged to be sterile.

Analysis of the Results

In addition to describing the variation observed on the individual characters, the effects of the method of subculturing the callus (simple subculture vs cloning), and the regeneration culture media (NK vs I medium) on those characters were tested using statistical means. The chi-square test was applied to the data of chromosome constitution, growth vigor, leaf color, esterase zymogram, chromosome association at MI in PMCs and pollen fertility. Analysis of variance was performed with the data on leaf shape and stomata number.

Results

Chromosome Constitution of Regenerated Plants

The root tips of some plants showed chromosome mosaicism in which hypodiploid cells were frequently observed which were identical to those occuring in the calluses (Ogihara and Tsunewaki 1979). In these cases, their modal karyotype was regarded as a representing karyotype.

The frequency of regenerated plants with different karyotypes is presented in Table 2. More than 80% of the regenerates from the simply subcultured calluses were diploid, while only 42% of those originating from the cloned calluses were diploid. The frequency of tetraploid plants (16L + 12S, 13.7% in total) was much higher than that of tetraploid cells (3%) in the stock cells (Ogihara and Tsunewaki 1979). Several new karyotypes were found among the regenerates. Four of these are shown in Figure 1. Three karyotypes had a similar chromosome constitution 1LL + 6L + 1M + 6S, which had never been seen in the stock callus. One extra-large (LL) and one medium (M) chromosome in the karyotypes were produced by the following translocation between two large (L) chromosomes: (I) translocation of a segment of the long arm of L_2 to the long arm of L_3 chromosome, (II) similar translocation of a larger segment than the first case, of the long arm of L_2 to that of L_3, and (III) translocation of a segment of the long arm of L_1 to the long arm of L_2 chromosome. Among the regenerates from cloned calluses, more new karyotypes, i.e., 7L + 1M + 6S, 15L + 12S, 16L + 11S, and 14L + 2M + 12S were observed, all of which are already known to occur in the cloned calluses. The M chromosome(s) included in these karyotypes was derived from deletion of an L_2 chromosome.

Comparison between Culture No. 2 and 4 should reveal the effect of the two methods of callus subculture (simple subculture vs cloning) on the karyotype of the regenerates (Table 1). The differences between them are significant at the 1% level ($x^2 = 59.0$, df = 5).

Cultures No. 1 and 3 were compared to reveal the effect of the redifferentiation media (NK vs I) on the karyotype of the regenerates. Tetraploids were found more frequently in Culture No. 1 than in No. 3. This difference is significant at the 1% level ($x^2 = 14.0$, df = 3).

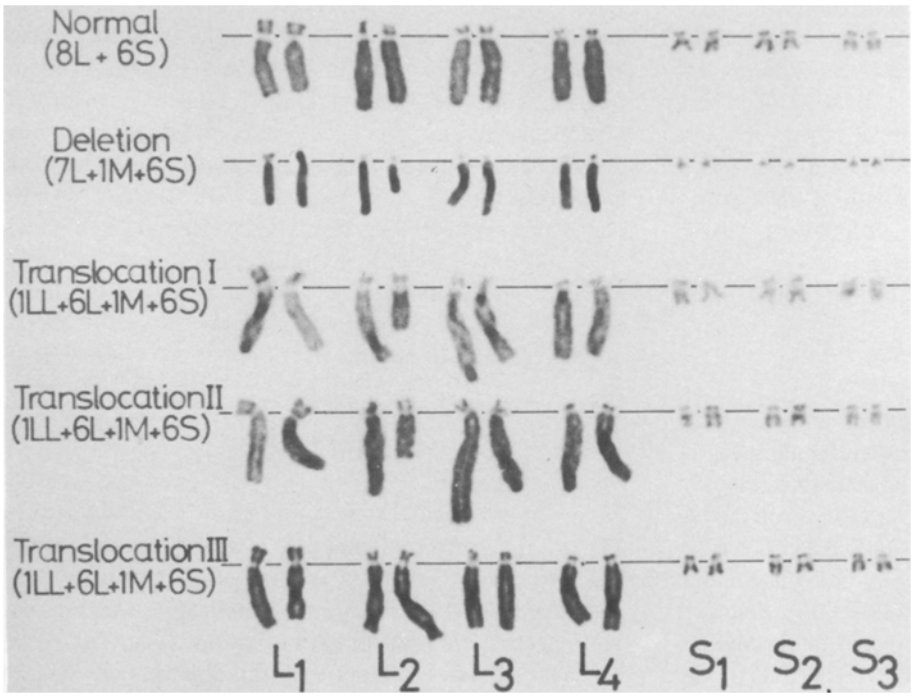

Fig. 1. Karyotypes found among regenerated plants. From top to bottom: 8L + 6S (normal), 7L + 1M + 6S (deletion of a segment of the long arm of L_2), 1LL + 6L + 1M + 6S (translocation I from L_2 to L_3), 1LL + 6L + 1M + 6S (translocation II from L_2 to L_3), 1LL + 6L + 1M + 6S (translocation III from L_1 to L_2). For details, see text

Table 2. Frequency in per cent of the regenerates with different karyotypes

Culture No.	No. plants observed	Karyotype						
		7L + 6S	7L + 1M + 6S	1LL + 6L + 1M + 6S	8L + 6S	15L + 12S	14L + 2M + 12S	16L + 12S
1	81	0	0	0	82.7	0	0	17.3
2	60	0	0	11.7	88.3	0	0	0
3	100	1.0	0	3.0	95.0	0	0	1.0
Subtotal of 1-3	241	0.4	0	4.1	89.2	0	0	6.2
4	147	0	20.4	0	42.2	2.0[a]	9.5	25.8
Total	388	0.3	7.7	2.6	71.4	0.8	3.6	13.7

[a] One hypotetraploid (16L + 11S) is included

Growth Vigor

In total regenerates, 71% of the plants showed vigorous growth, while 28% grew fairly well and 1% grew poorly. About 75% of the plants belonging to the normal diploid karyotype (8L + 6S) grew vigorously, while only 59% of the plants with aberrant karyotypes showed vigorous growth. A chi-square test revealed that the difference between the two classes of karyotypes (normal diploid vs others) on growth vigor is significant at the 1% level ($x^2 = 10.7$, df = 2).

The two methods of subculture seemed to have effected differences in the growth of the restored plants: about 20% of the regenerates in Culture No. 2 and about 54% of those in Culture No. 4 showed subnormal vigor (grade 1 or 2). The results of a chi-square test with the data on these two cultures revealed that this difference is significant at the 1% level ($x^2 = 15.4$, df = 2). This indicates that the proportion of subnormal regenerates was increased by cloning the calluses. Redifferentiation media did not seem to have affected the growth of the regenerates ($x^2 = 0.0$, df = 2, using the data of Culture Nos. 1, 3).

Leaf Shape

The regenerates showed great variation in leaf shape. Two leaf shape indices, i.e., length/width and length/thickness were adopted in order to clarify these leaf variations. In three cultures (Nos. 2-4), leaf shape indices of the karyotypic aberrants of the diploid (1LL + 6L + 1M + 6S and 7L + 1M + 6S) could be studied in comparison with those of the normal diploid (8L + 6S). No effect of chromosomal aberration was detected in the diploid level. Tetraploids (16L + 12S and 14L + 2M + 12S) had broader and thicker leaves than the diploids (8L + 6S and 7L + 1M + 6S) in the two cultures, No. 1 and 4. Analysis of variance indicated that their differences are significant at the 1% level ($F_{1, 208} = 40.9$ for L/W, and $F_{1, 208} = 56.7$ for L/T). The diploids (8L + 6S) in Culture No. 2 had slightly more slender and thinner leaves than those in Culture No. 4. These differences in both leaf shape indices were significant at the 1% level for L/W and at the 5% level for L/T, indicating effects of the subculture methods on leaf shape. No differences concerning both indices were observed between the normal diploids in Culture No. 1 and No. 3. Thus, the redifferentiation medium did not affect the leaf shape of the regenerates.

Leaf Color of Regenerated Plants

About 70 and 10% of the regenerated plants had normal and pale green leaves, respectively. The three other classes, i.e., pale yellow, red green and red remained as minor fractions (in total, 7.3%). Albino and partly green striped plantlets were also produced. All of the 27 albinos were derived from cloned calluses, and when transferred to a greenhouse all of them withered. Of the five striped plants regenerated, four plants were derived from No. 2 or 3 and one from No. 4 culture. The karyotype of the former four plants was 8L + 6S, and that of the latter was 7L + 1M + 6S. No effect of ploidy, subculture method or redifferentiation medium on leaf color was detected.

Stomata Number

The mean number of stomata observed per 0.25 mm^2 leaf area varied from 10.1 of the tetraploid (16L + 12S) in Culture No. 4 to 17.1 of the diploid (8L + 6S) in No. 2. The tetraploid, including the aberrant type (14L + 2M + 12S), had fewer stomata than the diploid, including its aberrant types (1LL + 6L + 1M + 6S and 7L + 1M + 6S). The pooled data revealed that the difference is significant at the 5% level, with $t_{df = 285} = 7.84$. No other factor,

such as method of subculture (Culture No. 2 vs No. 4), redifferentiation medium (Cult. No. 1 vs No. 3) caused a significant difference at the 5% level, at either the diploid or tetraploid levels.

Esterase Zymogram

Esterase zymogram observed in the leaves and roots of the regenerates are shown in Fig. 2. Five types (I-V) of zymograms were found in the leaves. The type III zymogram, that had four highly active bands (3-6), four minor bands (10-13) and four faint bands (2, 7-9), was obtained from the parental plant and most of the regenerates. The type II zymogram lacked three middle faint bands (7-9). The type I zymogram had only four major bands. The type IV zymogram had one additional faint band (I) and type V one more additional band (14) to type III. The most frequent of the regenerates from the simply subcultured calluses (Culture No. 1-3) was type III; type I occurred the most frequently among the regenerates from the cloned calluses. No effect was attributed to their ploidies. Using a chi-square test, no significant effect of different ploidies (2n vs 4n), including their aberrants, on the zymogram could be found at the 5% level (x^2 = 4.80, df = 4). Thus, it is concluded that autotetraploidization generally gives no effect on the zymogram. Comparison of the zymogram frequencies between Culture No. 2 and 4 revealed that the effect of subculture methods is significant at the 5% level (x^2 = 9.85, df = 3), while that between Culture No. 1 and 3 indicated that the effect of the redifferentiation medium was not significant at the 5% level (x^2 = 3.88, df = 4).

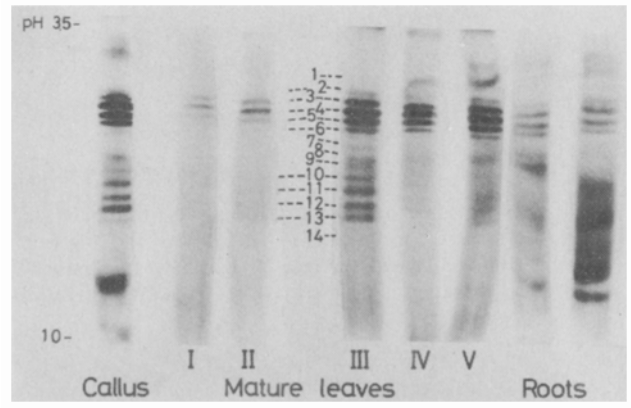

Fig. 2. Esterase zymograms observed in leaves and roots of the regenerates, compared to that of the stock callus (left)

Chromosome Association at Metaphase I in PMCs

Haworthia plants occasionally show chromosomal aberrations at meiosis (Brandham 1976). However, the original plants of *H. setata* showed no cytological abnormality in either somatic or meiotic cells, and formed seven normal bivalents (7_{II}) at MI of PMCs. On the other hand, regenerated plants revealed a wide variation of chromosome association as shown in Fig. 3 and Table 3. In eudiploids (8L + 6S), most PMCs (92%) formed seven bivalents, but

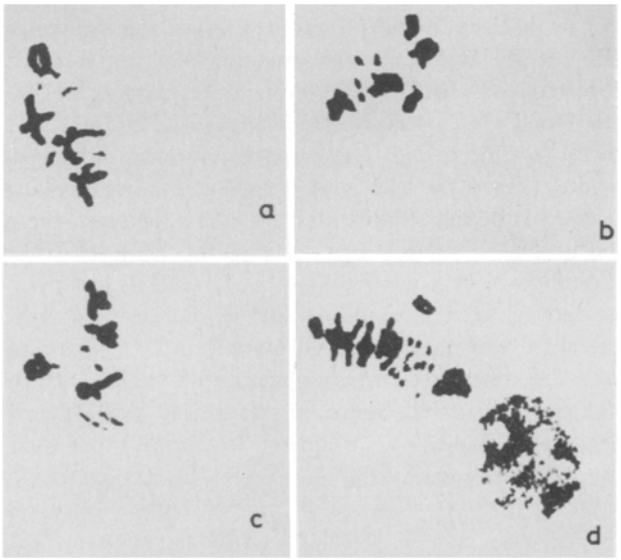

Fig. 3a-d. Chromosome associations at metaphase I of regenerated plants; **a** 7_{II} of 8L + 6S plant; **b** $2_I + 6_{II}$ of 8L + 6S plant; **c** 7_{II} of 1LL + 6L + 1M + 6S plant (type I); and **d** $3_{IV} + 7_{II} + 2_I$ of 16L + 12S plant

Table 3. Mean chromosome association at MI of PMCs observed in regenerates

Culture No.	Karyotype of root tip cell	No. plants observed	No. cells observed	Mean chromosome association								Freq. of PMCs showing 7″
				I	II	III	IV	V	VI	VII	VIII	
1	8L + 6S	33	1044	0.36	6.62	0.02	0.10	0.0				76.2
1	16L + 12S	10	138	1.78	4.62	0.46	3.30	0.06	0.10	0.03	0.06	–
2	8L + 6S	3	170	0.38	6.51	0.04	0.11	0.01				71.8
2	1LL + 6L + 1M + 6S	3	32	0.81	5.56	0.19	0.41					21.9
3	8L + 6S	24	676	0.55	6.54	0.03	0.08					69.2

some showed univalents (ca. 0.4 univalents/PMC). A few cells formed multivalents; namely, 0.03 trivalents and 0.10 quadrivalents per PMC. Plants with translocation and tetraploids more frequently formed univalents and multivalents than expected. A chi-square test revealed that the differences on chromosome association between the two classes of karyotypes, i.e., eudiploid (8L + 6S) vs eutetraploid (16L + 12S), and normal diploid (8L + 6S) vs translocation heterozygote (1LL + 6L + 1M + 6S) were both significant at the 1% level (x^2 = 6024.1, df = 7 and x^2 = 63.4, df = 4, respectively). Normal diploids in Culture No. 3 formed more univalents than the diploids in Culture No. 1; their difference was significant at the 1% level (x^2 = 72.5, df = 3).

Pollen Fertility

More than 90% of the pollen grains were morphologically normal in the original plant (Table 4), but some regenerated plants became partially sterile (60-90% pollen fertility) or partially fertile (40-60%). Even in the eudiploids (8L + 6S), there was some variation in pollen fertility. The frequency of fully fertile plants was highest in Culture No. 1 (74%), followed by Culture No. 2 (54%), and lowest in Culture No. 3 (27%). All translocation heterozygotes (karyotype 1LL + 6L + 1M + 6S) showed varying degrees of pollen sterility; the difference between them and normal diploids is significant at the 1% level (x^2 = 12.1, df = 2). It is noteworthy that tetraploids had a pollen fertility similar to that found in the diploids: their difference was not statistically significant (x^2 = 1.01, df = 2). The two redifferentiation media (NK vs I) seemed to have selected a difference in the pollen fertilities of regenerated plants. A chi-square test showed that their difference between Culture No. 1 and No. 3 is significant at the 1% level (x^2 = 54.2, df = 2).

Plants with Chromosomal Aberrations in Pollen Meiosis

In addition to the variants mentioned above, more variant regenerates with chromosomal aberrations were found from observations of PMC.

(i) Reciprocal translocation: Three plants with the non-reciprocal translocation belonging to the first type of translocation mentioned above had observable pollen meiosis and pollen fertility. They formed quadrivalents, 0.29, 0.29 and 0.64 per cell, and their pollen fertilities were 62, 40 and 37%, respectively. Therefore, diploid

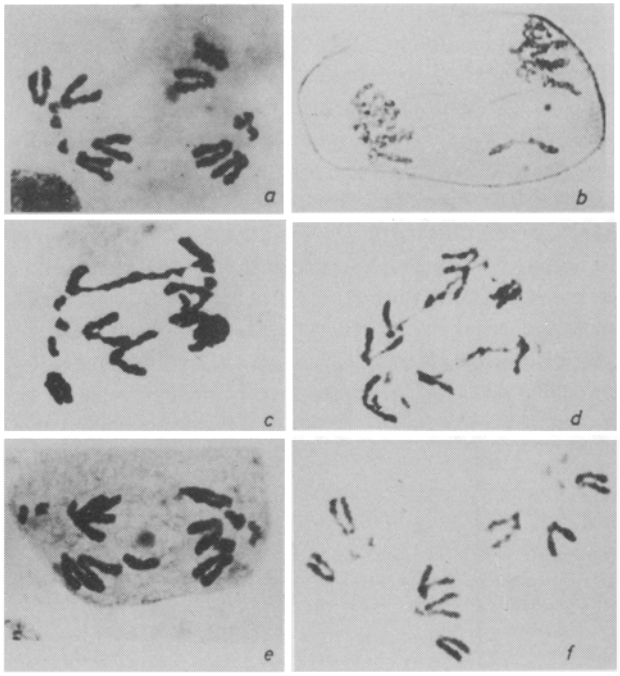

Fig. 4a-f. Chromosome segregation at anaphase I of diploid pollen meiosis; **a** normal segregation (7-7); **b** bridge and fragment; **c** bridge; **d** double bridge in one cell; **e** laggard; and **f** irregular 8-6 chromosome segregation

Table 4. Pollen fertility of the regenerates

Culture No.	Karyotype	No. plants observed	Relative frequency (%)			Mean pollen fertility (%)
			Fully fertile (90%)	Partially sterile (60-90%)	Partially fertile (40-60%)	
1	8L + 6S	50	74	22	4	91.5
1	16L + 12S	13	69	31	0	91.1
2	8L + 6S	11	55	36	9	74.2
2	1LL + 6L + 1M + 6S	3	0	33	67	46.0
3	8L + 6S	37	27	51	22	79.1
3	1LL + 6L + 1M + 6S	1	0	100	0	62.4

plants with more quadrivalents per cell than 0.30 were regarded to have reciprocal translocation since no aberration was detected in their root tip cells. In total, three plants with reciprocal translocation were regenerated. One of them was derived from Culture No. 1, another from No. 2 and the other from No. 3. They formed quadrivalents, 0.63, 0.33 and 0.38 per cell, and showed 79, 58 and 58% of pollen fertility.

(ii) Paracentric inversion: As shown in Fig. 4, many aberrations were observed in anaphase I of pollen meiosis, for example, bridge, bridge and fragment, lagging chromosomes, and abnormal chromosome segregation at AI. Three plants (two in Culture No. 1 and one in No. 3) showed bridge and fragment at AI of PMCs. They were regarded as plants that carried paracentric inversion, not U-type exchange, because the length of their fragments was almost constant at AI.

(iii) Sub-chromatid aberration: Some plants formed only bridges which were not fragments at anaphase I of PMCs. Three plants were considered to show sub-chromatid aberrations (Brandham 1970; Vig 1970). This sub-chromatid aberration was observed in a total of six plants at AI of PMCs (two in Culture No. 1 and four in No. 3).

Discussion

Chromosome Variability in Regenerated Plants and its Cause

There have already been some reports on the regeneration of plants with new karyotypes from cultured tissues: aneuploids in ryegrass (Ahloowalia 1976), trisomics in tobacco (Nishiyama and Taira 1966), a diploid carrying one isochromosome in lily (Sheridan 1974), hypotetraploid (4n-1) in *Haworthia setata* (Yamabe and Yamada 1973), and plants carrying chromosome rearrangements in *Crepis capillaris* (Sacristán and Wendt-Gallitelli 1971) and *Nicotiana sylvestris* (Maliga et al. 1979). All these karyotypes have also been observed in callus cells, excluding those from tobacco.

In the present investigation seven new karyotypes were found in the regenerates of *Haworthia setata*, four of which could be observed in callus cells; the other three, all carrying non-reciprocal translocation between different chromosome segments, were never observed in callus cells.

Chromosome variability among regenerated plants have been studied by several workers. Their studies showed that regenerated plants exhibited less chromosome variabilities than the original callus cells (Sacristán and Melchers 1969; Orton 1980). Therefore, it is evident that selection in favor of specific chromosome constitutions is operating during the course of plant regeneration from calluses. The intensity of the selection seems to vary depending on the materials used: very strong selection for euploids in common wheat and carrot (Shimada et al. 1969; Mok et al. 1976), moderate selection in tobacco, durum wheat and potato (Novák and Vyskot 1975; Bennici and D'Amato 1978; Shepard et al. 1980) and almost no selection in tobacco (Ogura 1976).

In the present material, selection for eudiploid and tetraploid plants was rather strong, although a few plants with abnormal karyotypes (deletions and translocations) were produced. However, chromosome chimerism was detected in many regenerates of *Haworthia*, that is, a few tetraploid or aneuploid cells were found among many eudiploid cells in the root tips of many diploid regenerates.

Chromosome variabilities observed in calluses and regenerated plants are shown in Fig. 5 for various standard deviation values in chromosome number. About 33% of the calluses revealed high chromosomal stability with small standard deviations (0-0.5). It is noteworthy that 16% of the calluses showed high chromosomal variability (their standard deviations were larger than 7.0). The standard deviation of the stock callus was 4.25. On the contrary, about half of the regenerates showed high chromosomal stability. On a whole, the regenerates showed slightly higher stability of chromosome number than the calluses: the difference was significant at the 1% level ($x^2 = 55.4$, df = 4). It should be emphasized, however, that some plants showed extreme chromosome variability with standard deviations higher than 5. These results agree with those of Sacristán and Melchers (1969) and Orton (1980). The relationship between chromosome variabilities of the calluses and the regenerated plants is shown in Fig. 6. The correlation coefficient between the standard deviations of their chromosome number was 0.54, which is significant at the 1% level. Such a relationship was previously reported by Heinz and Mee (1971) in sugarcane. Consequently, it is concluded that chromosome variation observed in the root tips of the regenerates is related to that of the calluses from which they originated.

As to the chromosome variability in regenerated plants, mixoploidy or chromosome chimerism was reported in monocotyledons, such as oats (Cummings et al.

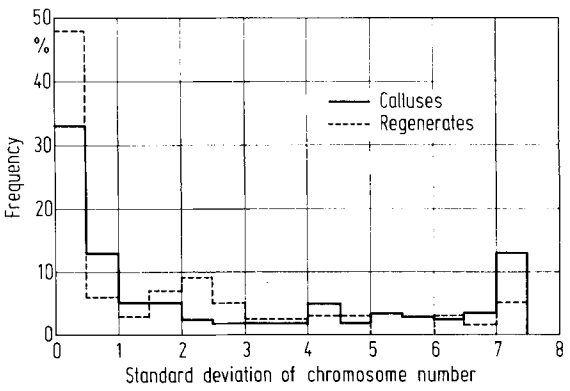

Fig. 5. Frequency of calluses and regenerated plants of Culture No. 4 for different standard deviation ranges of chromosome number

1976), barley (Mix et al. 1978), durum wheat (Bennici and D'Amato 1978), and lily (Bennici 1979) as well as in dicotyledons, such as tobacco (Sacristán and Melchers 1969; Novák and Vyskot 1975; Ogura 1976), *Lycopersicum peruvianum* (Sree Ramulu et al. 1976), and *Haworthia* (Yamabe and Yamada 1973; present investigation).

The following three possibilities can be considered with regards to the origin of regenerates with novel karyotypes: (1) Regeneration from single callus cells (Backs-Hüseman and Reinert 1970; Malnassy and Ellison 1970; review of D'Amato 1977). This process should give rise to only chromosomally uniform plants, unless the cell contains gene(s) for chromosome chimerism (Ogura 1976). (2) Adventitious shoots and/or roots originated from multicellular initials (Sree Ramulu et al. 1976; Mix et al. 1978; Bennici 1979), (3) New karyotypes are produced during morphogenesis in the meristematic tissues of the regenerates resulting in their mixoploidy (Orton 1980). As mentioned above, the chromosomal variability of the calluses reflected to some extent that of the regenerates (Fig. 6). Furthermore, some alterations of the modal karyotype took place during the course of plant regeneration, as shown in Table 5. From these facts, the two possibilities (1) and (3) are less likely to be the cause of chromosome chimerism observed in regenerated plants.

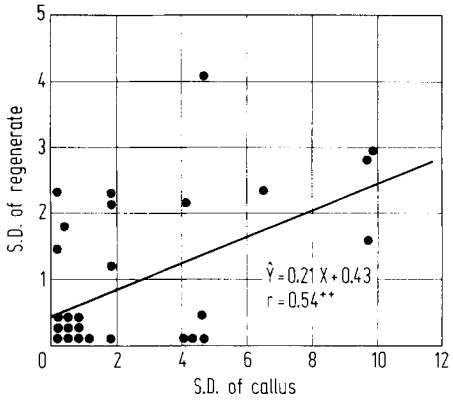

Fig. 6. Relationship between the standard deviations (S.D.) of the chromosome number of the calluses and of the regenerates

Table 5. Relationship between the modal karyotype of the regenerates and that of the calluses from which they originated

Relation	No. plants examined (%)
Same	19 (73.1)
Different	
Duplication (2x → 4x)	3 (11.5)
Reduction (4x → 2x)	1 (3.9)
Other alterations	3 (11.5)
Total	26 (100)

Also, they can not account for the origin of regenerated plants with new karyotypes which were never observed in callus cells. From these considerations, the second possibility is the most probable, that is, the stock callus used in the present investigation consisted of a heterogeneous cell population, and it had several meristematic pockets (Kaul and Sabharwal 1972; King et al. 1978) which were also comprised of karyotypically different cells in various proportions. Thus, shoot and/or roots formed from some pockets, particularly in cloned calluses, might have come to show different chromosomal constitutions, including chromosome chimerism, from the modal karyotype.

The relationship between the chromosome variabilities of somatic and meiotic cells are shown in Fig. 7, where the standard deviation of chromosome number and frequency of cells show 7_{II} bivalents at MI of PMCs. The correlation coefficient between them was −0.14, which is not statistically significant. The relationship between the chromosome variabilities observed in the root tip cells of the regenerates and their pollen fertilities was also analyzed. The correlation coefficient between them was

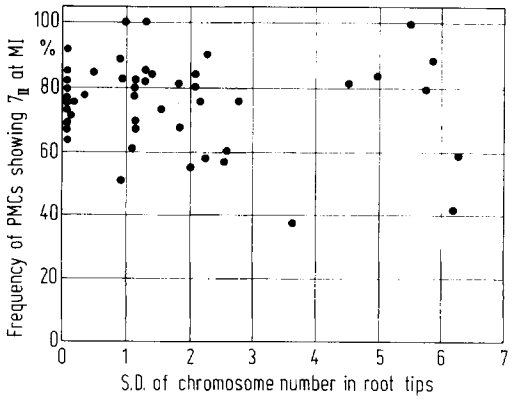

Fig. 7. Relationship between the chromosome variability, expressed as the standard deviation (S.D.) of the chromosome number of the root tip cells, and the meiotic regularity of PMC's in regenerated plants

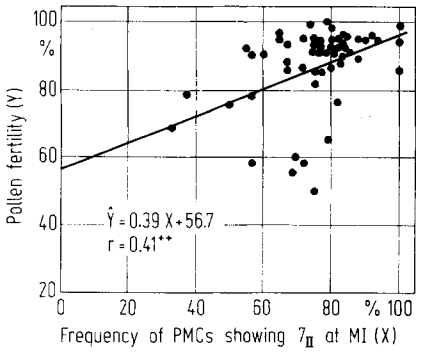

Fig. 8. Relationship between the meiotic regularity of the PMC's and the pollen fertility in the regenerates

−0.15, i.e., it is also not significant. On the other hand, the regularity of chromosome association at MI of PMCs (expressed in per cent PMCs with 7_{II}) and pollen fertility of the regenerates showed a correlation coefficient of 0.41, that is significant at the 1% level (Fig. 8). All these facts demonstrate that chromosome variabilities of callus cells were carried over to the somatic cells of the regenerates, but not to their generative cell lines. This result is contradictory with the findings obtained with tobacco (Zagorska et al. 1974; Ogura 1976).

Morphology of the Regenerates in Relation to their Chromosome Constitutions

There have been several reports on the phenotypes of regenerated plants from cultures with respect to their chromosome constitutions: Tetraploid regenerates of tobacco (Murashige and Nakano 1967), and n to 5n regenerates of rice (Nishi and Mitsuoka 1969) showed some phenotypic anomalies. Aneuploid regenerates of tobacco (Sacristán and Melchers 1969; Ogura 1978), sugarcane (Heinz and Mee 1971), *Lycopersicum peruvianum* (Sree Ramulu et al. 1976), and ryegrass (Ahloowalia 1976) also showed wide morphological variations. More remarkably, a rearrangement of a chromosome produced in *Crepis capillaris* (Sacristán and Wendt-Gallitelli 1971) was closely associated with abnormal morphology. Recently, Shepard et al. (1980) were able select many regenerates showing anomalies on several horticultural characters from protoplast-derived clones. They reported some of these anomalies as being associated with chromosomal disturbance and others showed wide variations regardless of their normal karyotype.

In the present investigation, a larger number of tetraploids were obtained from the NK medium than from the I medium, while, plants with structural changes of chromosomes (deletion and translocations) were selected in a larger number from the I medium than from the NK medium. How the difference of culture media influences the chromosome constitutions of the regenerates is unclear, but these alterations in chromosome constitutions somewhat affected the morphology of the regenerated plant. Changes in ploidy level caused alteration in growth vigor, leaf morphology, stomata number, and, of course, chromosome association at MI in PMCs, but no effects were detected on leaf color, esterase isozyme pattern, and pollen fertility. On the other hand, changes in chromosome structure at the diploid level (deletion and translocations) brought about no alterations in morphology of vegetative organs, but a marked effect was exerted on chromosome association at MI in PMCs and pollen fertility, as expected. Aneuploids and chromosomally chimeric plants did not reveal any special anomalies, which is in contrast to the phenotypic anomalies observed in chromosomally chimeric regenerates from tobacco (Ogura 1978). Therefore, it can be said that *Haworthia* plants are relatively stable in plant morphology when chromosome constitution is changed.

Comparison Between Chromosome Variations Selected Through Tissue Culture and Those Found in Nature

Field collections of *Haworthia* contain many chromosomal variants (Brandham and Johnson 1977). This is mainly due to vegetative propagation and occasional outbreeding in this plant, such as aneuploids in *Haworthia limifolia* (3n-2), *H. reinwardtii* (4n-2) and *H. tesselata* (6n-2) (Riley and Mukerjee 1962; Riley and Majunder 1966), and chromosomal rearrangements, including deletions, duplications, paracentric inversions (not detected in *Haworthia*), pericentric inversions and interchanges in the tribe *Aloineae* (Brandham 1976). Furthermore, meiotic anomalies such as E-type bridge (Brandham 1969), sub-chromatid aberrations (Brandham 1970; Vig 1970), and U-type exchange (Brandham 1970) were also observed.

Similar chromosomal variants were obtained in the present investigation from callus cultures of *Haworthia setata*, that is, aneuploids (2n-1 and 4n-1), paracentric inversions (three plants), and reciprocal (three plants) and non-reciprocal translocations (three types; in total 10 plants) and sub-chromatid aberrations (six plants). It should be emphasized that some remarkable differences existed between the regenerates from culture and the plants collected in nature. The first is a higher frequency of diploid plants carrying the deletion of a whole chromosome (one per 388 plants) or chromosome segment (30 per 388) in the regenerates. No such frequency was found among 597 diploid plants collected in nature (Brandham 1976). This might show that diploid plants carrying such chromosome deletions have some selective disadvantage in a natural environment. The second is the breaking point of chromosomes in the case of deletion and interchanges. Brandham (1976) critically analyzed the 51 interchanges found in the *Aloineae*. Of 102 breaks in these interchanges, 30 were at the centromere and 72 were at other positions, which indicated a large departure from randomness in favor of the centromere breaks. A similar tendency was reported in *Haplopappus* (Jackson 1965) and *Gibasis* (Jones 1974). In the present materials, all breaks found in the cultured regenerates were located in the chromosome arms, and none in the centric region. All translocations reported by Sacristán and Wendt-Gallitelli (1971) in *Crepis capillaris* and by Maliga et al. (1979) in *Nicotiana sylvestris* seem to have breaks in non-centric regions. An iso-chromosome reported by Sheridan (1974) in *Lilium longiflorum* is the only exception to this. From these results, we may conclude that chromosome breaks selected through callus cultures may differ from those occurring in nature in their origin and/or by their mechanism for maintenance.

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