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In Vitro Propagation and Chromosome Doubling of a *Triticum crassum* x *Hordeum vulgare* Intergeneric Hybrid*

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Summary. In vitro culture of inflorescence tissue of a *Triticum crassum* (6x) x *Hordeum vulgare* cv. 'Bomi' (2x) intergeneric hybrid resulted in the proliferation of totipotent callus from which plants were regenerated. Regeneration was also achieved from immature inflorescence callus of *T. crassum* but not from *H. vulgare*. *T. crassum* x *H. vulgare* regenerates had a somatic chromosome number of 28, identical to that of the original hybrid. Four chimeric plants with a partially doubled chromosome number were obtained by in vitro colchicine treatment of hybrid callus prior to induction of plant regeneration. All *T. crassum* regenerates had 35 chromosomes rather than the expected number of 42. Meiotic analysis of a 35-chromosome plant revealed an extremely abnormal meiosis which might be attributable to a complete disturbance in meiotic control system(s) including that of meiotic pairing.

Key words: *Triticum crassum* (6x) x *Hordeum vulgare* cv. 'Bomi' (2x)-Intergeneric hybrid – Inflorescence culture – Plant regeneration – In vitro colchicine treatment – Chromosome doubling

Introduction

Intergeneric hybrids in cereals have often been produced through the use of embryo culture techniques. Although the frequency of production of such hybrids is often low, viable hybrids have been obtained amongst the genera, *Triticum*, *Hordeum*, and *Secale* (Kruse 1967, 1973; Fedak and Armstrong 1980). Hybridization in the *Gramineae* has been utilized in a number of instances to accomplish interspecific and intergeneric gene transfers such as genes for disease resistance (Knott and Dvorak 1975). Such gene transfers have been possible only in hybrid combinations that conferred partial fertility to female gametes or in hybrids that could be converted into fertile amphiploids by conventional colchicine treatment. However, intergeneric hybrids are often sterile and fertile amphiploids have rarely been produced between either *Triticum* and *Hordeum* or *Hordeum* and *Secale*. If fertile, stable amphiploids were obtained in these crosses they could be used in the production of chromosome addition and substitution lines which would be of value in intergeneric gene transfer. Tissue culture techniques may have potential in developing such gene transfer systems.

We have recently obtained viable intergeneric hybrids between hexaploid *T. crassum* (or *Aegilops crassa*) and diploid *H. vulgare* cv. 'Bomi' through the use of embryo culture techniques (Fedak and Nakamura 1981). Chromosome pairing at the first meiotic metaphase of the F_1 hybrids was irregular and chromosome homoeology between the parental species was limited. Hybrid spikes were sterile and attempts to backcross the hybrids with *T. crassum* have thus far been unsuccessful. We have also initiated a study aiming at the development of tissue culture techniques applicable to cereal hybrids by utilizing hexaploid triticale as a model system. Morphogenic cultures were initiated from immature embryos (Nakamura and Keller 1981) and immature inflorescences (Nakamura and Keller, in prep.). The objective of this paper was to apply the potential techniques for in vitro cereal regeneration to the *T. crassum* x *H. vulgare* hybrid tissues as well as to both parental species. The application of in vitro colchicine treatment for the restoration of fertility in regenerates was also evaluated.

Materials and Methods

The intergeneric hybrid between *T. crassum* (Boiss.) Aitch. & Hensl. (2n = 6x = 42) and *H. vulgare* L. cv. 'Bomi' (2n = 2x = 14),

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and both parental species were grown in a growth cabinet maintained at a day/night temperature of 20/15°C in a 16 hr photoperiod under combined fluorescent and incandescent light (32,000 lux). Explants for callus induction and plant regeneration were obtained from young developing inflorescences (1-3 cm in length) from the hybrid as well as from the parental species. Excised inflorescences were surface-sterilized with 70% ethanol for 2 min, followed by immersion in saturated calcium hypochlorite for 5 min and then washed three times with sterile water. Inflorescences were divided into two sections and each section was further cut into small explants and cultured in liquid or on agar (0.8% Difcobacto) medium in 60 X 15 mm plastic petri dishes.

For callus induction and plant regeneration the basal medium initially employed by Kao (1977) was utilized with some modifications as described elsewhere (Nakamura and Keller 1981). The liquid and agar media were adjusted to pH 5.7 and 6.0, respectively, with 0.1 N KOH. The liquid medium was filter-sterilized and the agar medium was autoclaved for 17 min at 121°C and 1.1 kg/cm². For callus induction, 5 mg/L of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was added to both liquid and solid basal media, and the cultures were incubated at 24°C in 10 hr photoperiods under 500 lux of fluorescent light. After a 4-week induction period, the calli were subcultured at least twice at 4-week intervals on medium with 2 mg/L of 2,4,5-T and then transferred to a solid auxin-free medium in 60 X 20 mm dishes and incubated under 1,500 lux of continuous fluorescent light at 25°C. When regenerated shoots reached a length of 2-3 cm, they were transferred to half-strength basal medium in 15 X 2.5 cm glass tubes to stimulate root development and autotrophic growth of the regenerated plantlets. The plantlets were transplanted to Jiffy-7 peat pellets, placed in a mist chamber for approximately two weeks prior to potting and then maintained in a greenhouse.

To induce chromosome doubling, one hybrid callus which was subcultured three times on the solid subculture medium was transferred to the same medium supplemented with filter-sterilized colchicine at 20 mg/L and incubated at 10°C for ten days in darkness according to the method developed by Chen and Goeden-Kallemeyn (1979). The callus was then washed with the liquid basal medium and transferred to the regeneration medium and maintained under continuous fluorescent light (1,500 lux) at 25°C. For cytological evaluation of regenerates, root tips were pretreated with ice cold water for 24 hr and fixed with 1:3 acetic ethanol. Anthers were also collected and fixed. The materials were stained with Snow's solution (Snow 1963) and slides were prepared by the standard squash method.

Results

Callus Induction

Initiation of callus proliferation was observed within several days on liquid induction medium and in approximately one week on agar medium. Calli induced on agar medium were yellowish and compact, but those on liquid medium appeared very fragile and ceased to grow upon transfer to solid subculture medium. Callus induction rates on solid medium were 75% and 50% for explants from young inflorescences of *T. crassum* and the hybrid, respectively (Table 1). During the subculture period calli remained yellowish-white and callus growth was rather limited.

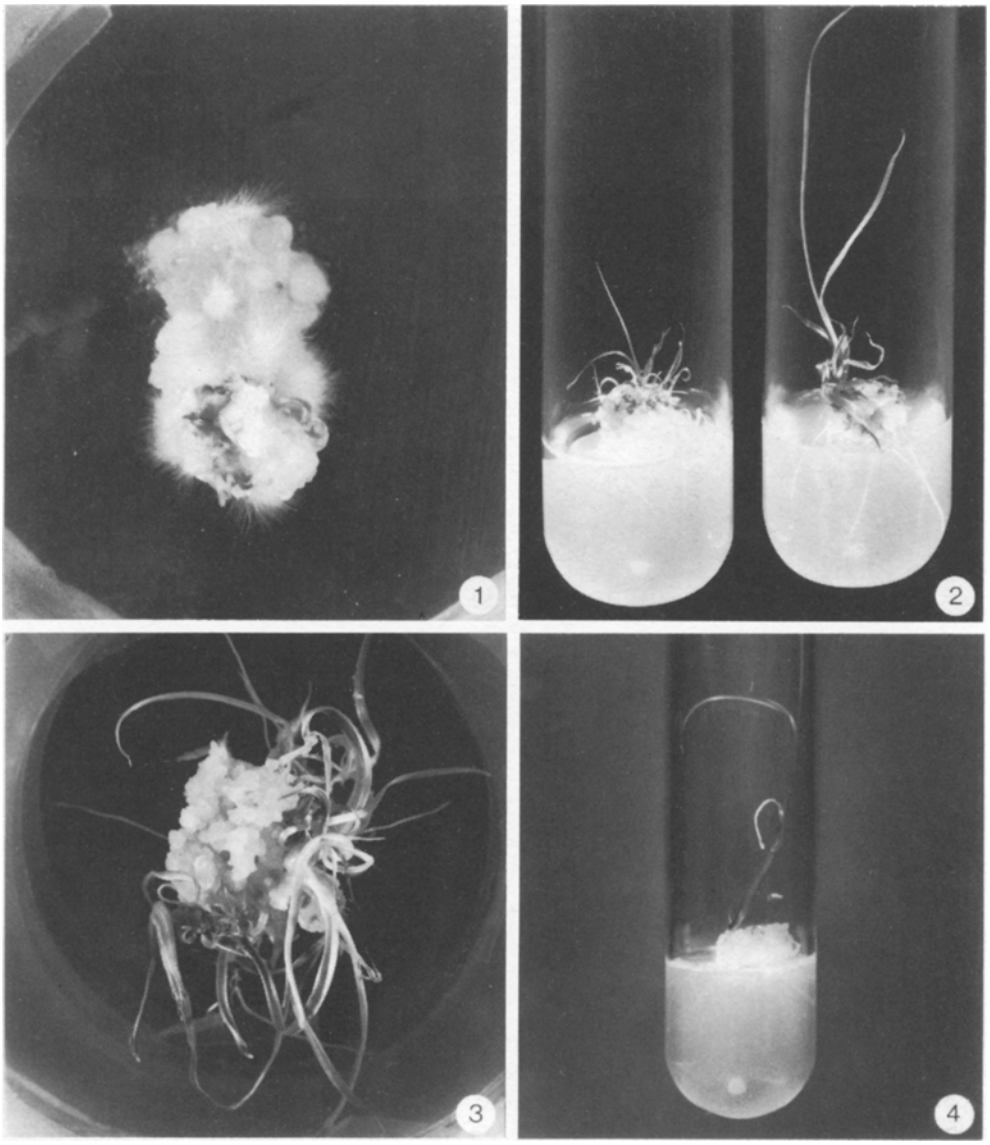
Table 1. Callus induction and plant regeneration from *Triticum crassum* and an intergeneric *Triticum crassum* X *Hordeum vulgare* cv. 'Bomi' hybrid

Donor	No. of explants cultured	No. of explants induced to callus	No. of explants regenerating plants	No. of plants regenerated[a]
T. crassum	8	6	2	52
F_1 hybrid (no. 4)	10	5	3	23
H. vulgare	12	8 (1)[b]	0	0

[a] In addition to the 52 green plants, more than 15 albino shoots were obtained from *T. crassum* calli; [b] Only one out of eight calli survived the first subculture

Plant Regeneration

Upon transfer to solid, auxin-free regeneration medium growth resumed and multiple shoot and root initiation occurred on the callus surface as early as one week of culture (Fig. 1). Shoot regeneration was usually followed by root regeneration. Those calli which regenerated roots first, rarely produced shoots. By the end of the four-week regeneration period, regenerated shoots developed vigorously and could be individually excised (Fig. 3). Proliferation of compact, yellowish calli usually occurred simultaneously with multiple shoot regeneration (Fig. 3). Individually excised shoots with attached calli were transferred to tubes containing half-strength basal medium (Fig. 4). During subsequent culture new shoots as well as new calli sometimes proliferated (Fig. 2). The calli were separated from the shoots and subjected to a second period of regeneration or subcultured on medium with 2 mg/L of 2,4,5-T. Even during subculture periods on the auxin-containing medium, multiple shoots occasionally formed. Calli which did not undergo shoot formation were also subcultured in the same way. Plant regeneration occurred in 25% of the *T. crassum* and 30% of the hybrid explants (Table 1). Fifty-two plants have been regenerated from the two original explant-derived *T. crassum* calli and 23 regenerates have been obtained from three original hybrid calli. All the hybrid regenerates were green. Although the majority of *T. crassum* regenerates were green, more than 15 albino shoots or anthocyanin-pigmented pink shoots were also identified. In the case of *H. vulgare* cv. 'Bomi', six inflorescences were cultured and eight calli (67% of the explants) were obtained. However, only one callus survived the first subculture and no plant regeneration was achieved.



Figs. 1-4. *T. crassum* callus regenerating shoots and roots (**1**); plantlets regenerating from *T. crassum* calli (**2**); *T. crassum* × *H. vulgare* hybrid callus regenerating shoots and roots. New callus was simultaneously proliferating during the regeneration period (**3**); *T. crassum* × *H. vulgare* hybrid plantlet cultured in a tube containing half-strength medium (**4**)

Cytological Evaluation of Regenerates

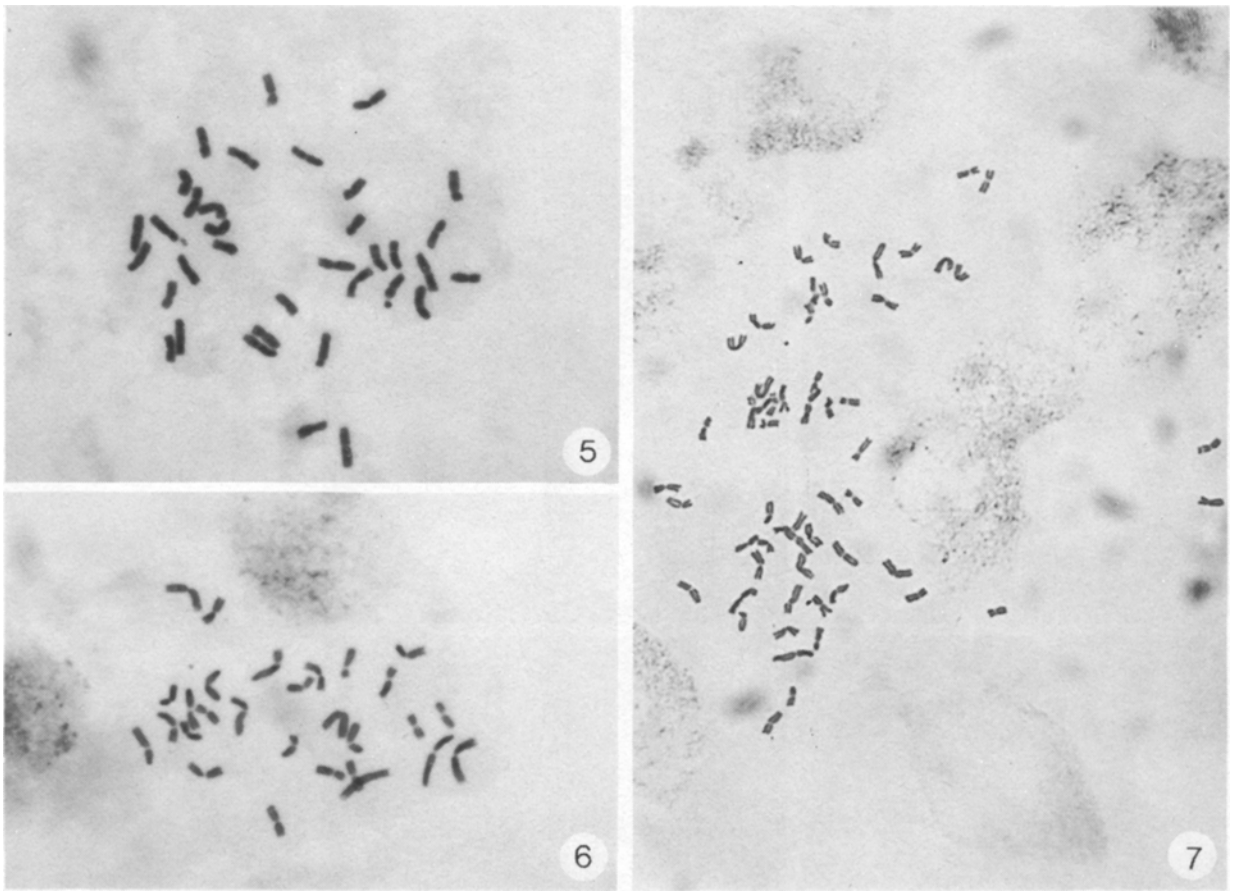
Cytological analysis of *T. crassum* regenerates revealed a somatic chromosome number of 35 with seven chromosomes missing from the original hexaploid number (Fig. 5, Table 2). Although chromosome counts were not obtained for each regenerate, all were completely sterile, suggesting that they were 'pentaploids'. Chromosome pairing at metaphase I of one regenerate was abnormal (Figs. 8, 9). The average pairing configuration at metaphase I was $0.07^{IV} + 1.78^{III} + 6.13^{II} + 17.13^{I}$ and the average chiasma frequency per sporocyte was 11.73. Reduction division at anaphase I was also abnormal and chromosome bridges and many laggards were observed (Figs. 10 and

Table 2. Chromosome numbers of regenerates from *Triticum crassum* and *T. crassum* × *Hordeum vulgare* hybrid

Regenerates		No. of plants with 2n = 28	No. of plants with 2n = 35	No. of plants with 2n = 28-56
T. crassum[a]	callus no. 1	–	5	–
	callus no. 2	–	4	–
F_1 hybrid (no. 4)[b]	callus no. 1	3	–	–
	callus no. 3	2	–	4

[a] These 9 plants plus an additional 43 plants, whose chromosome numbers were not studied, were completely sterile

[b] These 9 plants plus an additional 14 plants, whose chromosome numbers were not studied, were completely sterile



Figs. 5-7. Somatic metaphase of *T. crassum* regenerate (2n = 35), (5); somatic metaphase of *T. crassum* × *H. vulgare* regenerate (2n = 28), (6); somatic metaphase of chimeric amphiploid regenerated from colchicine-treated *T. crassum* × *H. vulgare* hybrid callus (2n = 56), (7)

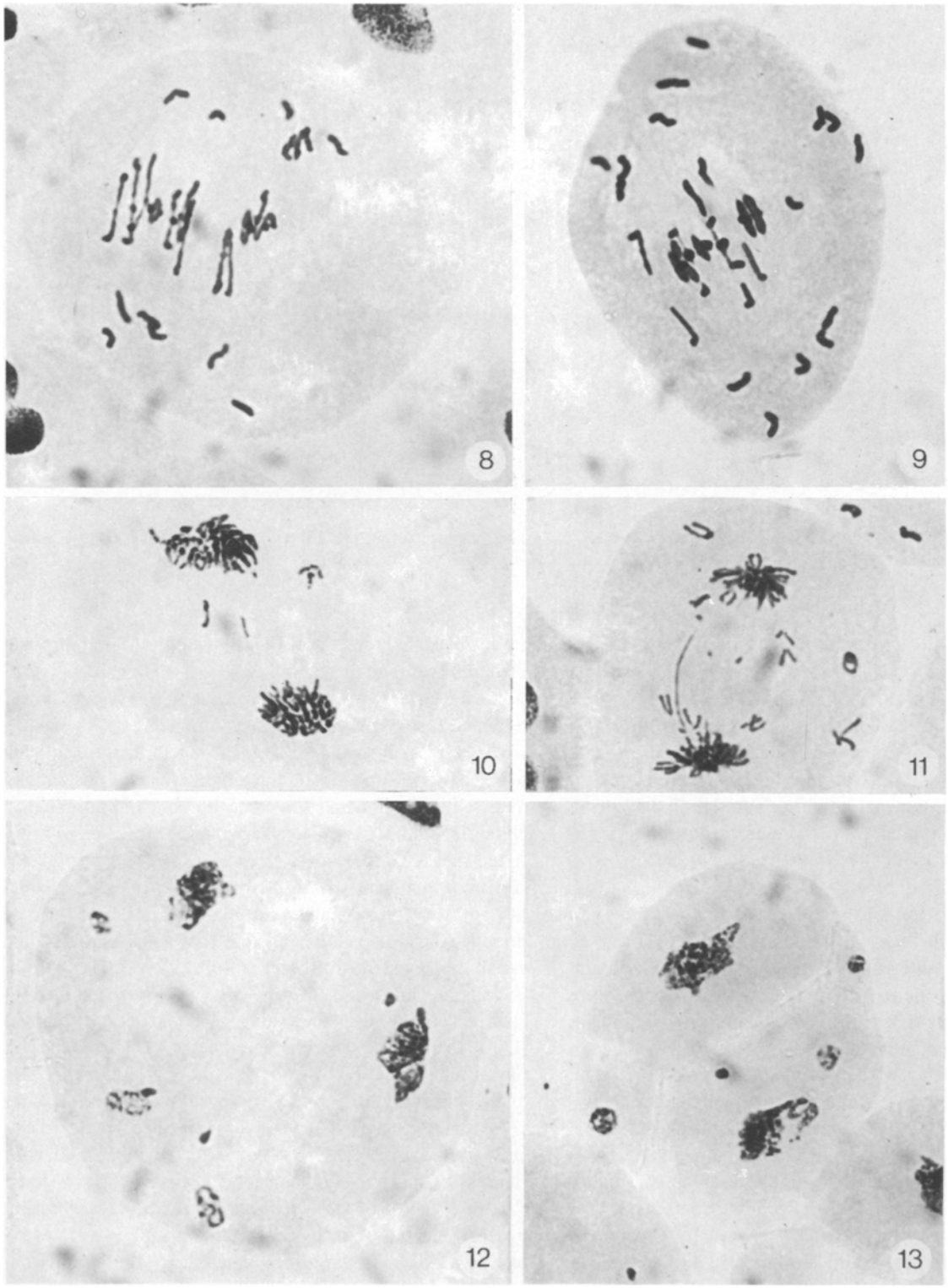
11). At telophase I, 94.5% of the sporocytes had 1 to 8 micronuclei (Fig. 12). Unusual cytokinesis was observed with many sporocytes producing more than two daughter cells at the end of the first division (Fig. 13). Somatic cells of regenerates derived from hybrid calli had 28 chromosomes, identical to the original hybrid (Fig. 6 and Table 2). Transfer of a single colchicine-treated callus to auxin-free medium resulted in the production of 10 plants, four of which were partial somatic chimeras with 28 and 56 chromosomes (Fig. 7, Table 2).

Morphology of Regenerates

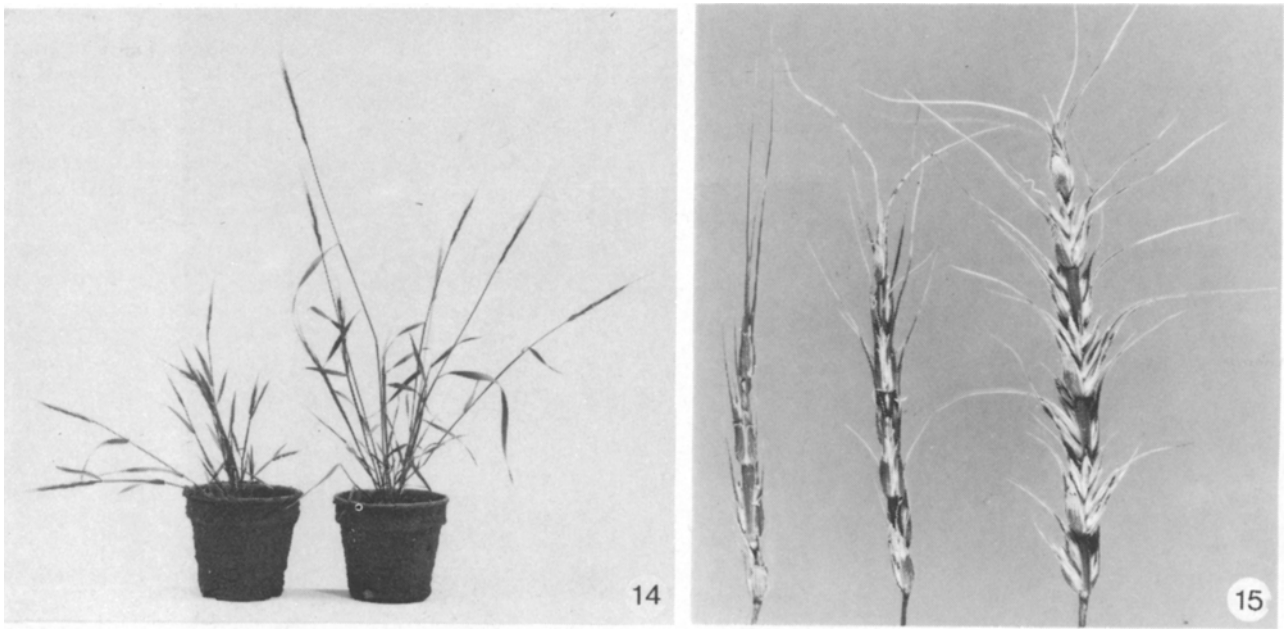
The general morphology of the regenerates from the hybrid tissue explants was similar to that of the original hybrid donor plant as previously described (Fedak and Nakamura 1981). However, they were shorter (approximately 45 cm) and had fewer tillers (Fig. 14). Spikes of the regenerates consisted of six to eight spikelets, or about half that of the original hybrid. The regenerate spikelets were also smaller. Chimeric plants regenerated from the hybrid were morphologically similar to the sterile regenerates. However, in such chimeric plants spike and spikelet morphology differed from other regenerates in that the glumes opened and ovules grew until abortion took place (Fig. 15). However, the chimeric plants did not set seeds. The *T. crassum* regenerates were shorter (approx. 20 cm in height) and had fewer tillers than the hexaploid donors (Fig. 14). The floret structure and size were similar to the original hexaploid but they were completely sterile. All *T. crassum* and hybrid regenerates did not require vernalization and flowered after transplanting to pots.

Discussion

We have been able to demonstrate that immature inflorescences are a suitable explant source for callus induction and subsequent plant regeneration in a *T. crassum* × *H. vulgare* hybrid (Table 1). Immature inflorescence tissue has also been utilized as donor tissue for sugarcane and corn propagation (Liu et al. 1972; Rice et al. 1979; Molnar et al. 1980). In the case of intergeneric hybrids,



Figs. 8-13. Meiotic analysis of *T. crassum* regenerates; **(8, 9)** meiotic metaphase; **(8)** meiocyte with $2^{III} + 8^{II} + 13^{I}$; **(9)** meiocyte with $10^{II} + 15^{I}$; **(10-13)** anaphase I to telophase I of *T. crassum* regenerate; **(10)** meiocyte with 5 laggards; **(11)** meiocyte with one chromosome bridge and many laggards; **(12)** two daughter cells with 5 micronuclei; **(13)** four daughter cells with 4 micronuclei



Figs. 14-15. *T. crassum* regenerate (2n = 35, L) and *T. crassum* × *H. vulgare* regenerate (2n = 28, R) **(14)**; (L-R). Spikes of regenerates; *T. crassum* (2n = 35), *T. crassum* × *H. vulgare* (2n = 28), *T crassum* × *H. vulgare* (chimeric plant) **(15)**

other explant sources have also been utilized for the induction of plant regeneration. Shumnyi and Pershina (1979) reported the in vitro propagation of barley × rye hybrids from shoot apices of lateral tillers. Orton (1979) induced regeneration in calli derived from immature ovaries of *H. vulgare* × *H. jubatum* hybrids. The clonal propagation of sterile intergeneric hybrids through tissue culture techniques as achieved in the present study and as observed by others could have potential value in introgressive breeding.

Plant regeneration was achieved from *T. crassum* and the *T. crassum* × *H. vulgare* hybrid but not from *H. vulgare* (Table 1). Orton (1979) reported that calli obtained from *H. jubatum* and its hybrids with *H. vulgare* were morphogenic, but those from *H. vulgare* were not. It has been known that perennial wild relatives have higher morphogenic capacity than annual cultivars (Tal et al. 1977). Orton (1979) has suggested the possibility of utilizing in vitro systems for transferring morphogenic capacity from perennial relatives to annual cultivars. Morphogenic capacity in hybrids and amphiploids is also under control of specific genome(s) and genome combinations as shown in studies with *Nicotiana* species by Cheng and Smith (1973) and Ogura and Tsuji (1977). Nakamura and Keller (1981) have recently suggested that a high morphogenic capacity in a hexaploid triticale cv. 'Welsh' may be ascribed to specific D genome chromosomes of common wheat which substitute for homoeologous rye chromosomes. Evaluation of the effect of various genome and chromosome constitutions upon morphogenic capacity could help facilitate the use of tissue culture in improving *Triticum* and related genera.

Regenerates from the *T. crassum* × *H. vulgare* hybrid retained a somatic chromosome number of 28 (Fig. 6), whereas all analyzed *T. crassum* regenerates were missing seven chromosomes from the original hexaploid complement (Fig. 5). Recently, a large number of spontaneous chimeric plants have been regenerated from newly initiated callus cultures of *T. crassum* × *H. vulgare* hybrids (Fedak unpublished data). Although a cytological evaluation of the hybrid callus cultures was not made in the present study, it is quite possible that some chromosome variability existed in these cultures. Prolonged culture periods are known to induce chromosome instability including polyploidy, aneuploidy, and structural changes (D'Amato 1977, 1978; Sunderland 1977). Fertile amphiploids might be obtained through polyploidization induced during culture of hybrid tissues if the amphiploid cells undergo morphogenesis and produce fertile tillers. However, such polyploidization is often associated with the loss of morphogenic capacity. Orton (1980) reported that in *H. vulgare* × *H. jubatum* hybrids the regeneration process selected against polyploid cells and to a lesser extent against cells exhibiting aneuploidy and structural changes. Complete selection against polyploidy in regenerates has also been reported by Shumnyi and Pershina (1979) in barley × rye hybrids. It is, therefore, possible that the isolation of spontaneous amphiploids through tissue culture techniques may not be effectively achieved in cereal hybrids.

The in vitro colchicine treatment of *T. crassum* × *H. vulgare* hybrid callus resulted in the production of partial chimeras (Fig. 7 and Table 2). Since neither spontaneous amphiploids nor sectorial chimeras were detected amongst regenerated plants from untreated calli, the partial chromosome doubling observed in the present study was most probably induced by the colchicine treatment. Orton and Steidl (1980) treated *H. vulgare* × *H. jubatum* hybrid calli with colchicine and subsequently identified various aneuploid plants amongst the regenerates.

Aneuploidy and chimerism were frequently detected amongst pollen mother cells; the complete sterility of the regenerates was attributed to meiotic breakdown caused by such chromosome instability. Since interspecific and intergeneric sterility is partly due to chromosomal and genic incompatibility between species (Stebbins 1958), fertility restoration in hybrids may not always be achieved by simply doubling the chromosome numbers. Barley × rye hybrids which so far have been produced (Kruse 1967, 1976; Thomas and Pickering 1979) were in fact completely sterile even after chromosome doubling. Intergeneric incompatibility may also be a factor in attempts to achieve the production of fertile *T. crassum* × *H. vulgare* hybrids. However, in vitro chromosome doubling would still be an important initial step towards fertility restoration in such hybrids. To realize this, rapidly growing and synchronized cell cultures should be treated with colchicine or other chemical agents prior to induction of plant regeneration. Such experiments are currently in progress.

The entire meiotic process in one *T. crassum* regenerate with 35 chromosomes was extremely abnormal (Figs. 8-13). An average univalent frequency was 17 per sporocyte, which was much higher than the expected number of seven or less based on studies of meiotic pairing in *T. crassum* polyhaploids (Shigenobu and Sakamoto 1977), *T. crassum* × *H. vulgare* hybrids (Fedak and Nakamura 1981), and in *T. crassum* (6x) × (4x) hybrids (Kihara 1949). It was thus concluded that chromosome pairing in this regenerate was suppressed even between homologous chromosomes. Whether the seven missing chromosomes belong to one entire genome, either D, D^2 or M^{cr}, or involve two or three genomes is unknown. However, the extremely abnormal meiotic behavior suggested that the meiotic control system(s) including that of meiotic pairing failed to function in this regenerate. Regenerates with 35 chromosomes were obtained from two calli derived from different inflorescences, thus suggesting that this type of chromosome loss may be reproducible in this species. Further tissue culture studies with immature inflorescences and/or immature embryos and possibly with haploid tissue may help to clarify the role of specific genomes or chromosomes in regeneration of aneuploids and in subsequent abnormal meiotic processes.

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