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Gynogenic plant regeneration from unpollinated flower explants of *Eragrostis tef* (Zuccagni) Trotter

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Abstract Tef [*Eragrostis tef* (Zucc.) Trotter] is the most important cereal in Ethiopia. In its wild relative *E. mexicana*, regeneration of six green plants resulted from culture of 121 non-pollinated immature pistils. In the allotetraploid crop species tef, however, only callus and root formation was obtained by this method. By contrast, immature spikelets and panicle segments of *E. tef* proved amenable to gynogenic plant regeneration. Upon step-wise optimization of the protocol, efficient plant formation was achieved in all three cultivars tested. In cv. DZ-01-196, culture of 1305 immature spikelets resulted in formation of 159 green plants. Flow cytometric analysis revealed (di)haploid, triploid, tetraploid and octoploid regenerants, from which the vast majority was tetraploid. Tef-breeding programs will likely benefit substantially from efficient generation of true-breeding plants.

Keywords Embryogenesis · Gynogenesis · Haploid · Ovary · Pistil

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Introduction

The development of efficient tissue culture techniques is imperative to facilitate breeding programs for the traditional, economically important Ethiopian cereal tef. Such contemporary technology could be valuable as pre-breeding tools, e.g. for germplasm preservation, distant hybridisation and genetic transformation approaches, or can be more or less directly implemented in conventional line selection by facilitating asexual multiplication, directed mutagenesis or production of doubled haploids. Tef is a strictly self-pollinating crop. Tef breeding has been based upon cross-pollination and line selection which resulted in a number of improved varieties. However, landraces still are widely grown in agriculture. So far, very little work has been done in tef biotechnology. Plant regeneration in vitro from roots, leaf bases and seeds (Bekele et al. 1995; Mekbib et al. 1997; Kebebew et al. 1998) has been reported. The efficiency of these published protocols is generally too poor for serious application in tef breeding, and results on culture of immature reproductive organs such as embryos or ovules are entirely missing so far. Moreover, there is thus far no method available for the generation of haploid or doubled haploid plants which may dramatically increase the efficiency of elite line selection. In many other crops, haploid or doubled haploid plants can be regenerated from cultured male gametophytes (isolated immature pollen) or gametophyte-bearing organs such as immature anthers, non-fertilised pistils, ovaries or ovules thereof. Gynogenic plant regeneration has been reported in a number of crops including cereals, e.g. barley (San Noeum 1976; Castillo and Cistué 1993), wheat (Zhu et al. 1981), rice (Zhou and Yang 1980; Asselin de Beauville 1980) and maize (Ao et al. 1982; Truong-André and Demarly 1984).

Our initial attempts to generate haploids or doubled haploids were based on culture of immature anthers or isolated pollen. However, only some androgenetic calluses were obtained which typically failed to undergo embryonic development and plant regeneration. So far only one albino tef plantlet was generated from isolated microspores

(unpublished results). Interestingly, when immature tef pistils were co-cultured with immature anthers or pollen to support androgenesis as was earlier reported for barley and wheat (e.g. Koehler and Wenzel 1985; Mejza et al. 1993), the pistils enlarged and some even formed callus. This preliminary observation encouraged us to embark on a detailed investigation using various explant types from tef inflorescences before pollination aiming at gynogenic development and plant regeneration. Here we present experiments which, for the first time, resulted in gynogenic plant regeneration from unpollinated pistils in the wild species *E. mexicana*, as well as from isolated spikelets or panicle segments of three *E. tef* cultivars.

Materials and methods

Plant material

Three tef [*Eragrostis tef* (Zuccagni) Trotter] cultivars, namely the landrace Fesho, and the widely grown improved varieties DZ-CR-37 and DZ-01-196 ($2n = 4x = 40$), as well as the phylogenetically related wild species *E. mexicana* ($2n = 6x = 60$) which is also strictly self-pollinating were used. Seeds of these genotypes were germinated in peat–soil mix in a glasshouse at about 26°C. Fertilizer Plantosan (Aglucon, Duesseldorf, Germany) was applied using 12 g per pot once after 3 weeks of potting. Immature panicles which had emerged from the flag leaf sheath as specified below were chopped into segments of about 5–7 cm. Surface sterilization was carried out using 1% sodium hypochlorite solution in 50 ml screw-capped plastic centrifuge tubes. The tubes were shaken for 15 min and then rinsed 3–4 times with sterile doubled distilled water.

Preparation and culture of explants

Pistils were dissected from the florets using a binocular microscope. Florets were detached from the spikelet and cut at the base. Then, the pistil along with the three stamens was pushed slowly towards the cut opening and upon its complete release the anthers were removed. Isolated pistils were grouped according to their size. The size classes were defined according to the developmental stages of pollen found in the same florets, i.e. small pistils corresponded to male meiosis until microspore stage (7–5 days before pollination), medium-sized pistils to bicellular pollen (4–3 days before pollination), and large pistils to tricellular pollen (2–1 days before pollination), respectively. About 10–15 pistils were cultured per 35-mm Petri dish. For inductive treatment at the beginning of pistil culture, the dishes were incubated at 4°C for 3 days, if not stated otherwise. MS (Murashige and Skoog 1962) and L3 medium (Jaehne et al. 1991) were used for *E. tef* and *E. mexicana* cultures, respectively. Both media were solidified with 0.3% Gelrite and supplemented with 9.2 μM 2,4-D, if not stated otherwise.

Detached spikelets and panicle segments of different developmental stages were distinguished according to the length of panicle emergence from the flag leaf sheath, i.e. 0–5 cm, 6–9 cm, 10–13 cm, 14–17 cm (corresponding to the early microspore up to the bi-cellular pollen stage) and 18–20 cm (corresponding to the tri-cellular pollen stage and the commencement of anthesis in the most basal flowers which were not used for culture). Furthermore, the panicles were divided into basal, central and top segments for panicle segment culture, whereas for spikelet culture, only the central segments were used. For temperature treatment prior to detached spikelet or panicle segment culture, the panicle segments were kept in tubes containing water and stored at 32°C for 24 h or at 4°C for 12, 24, 36 and 72 h, while non-pretreated segments were used as control. Detached spikelets and panicle segments were cultured in 6-cm Petri dishes with about 50 spikelets per dish containing MS medium solidified with 0.3% Gelrite and supplemented with 9.2 μM 2,4-D and 8.9 μM BAP, if not stated otherwise.

For induction of gynogenic tissue, the cultures were incubated at 24 ± 2°C either in the dark or with 16 h photoperiod for about 1 month. Then, the entire explants along with the embryonic tissue generated were transferred to MS medium (Murashige and Skoog 1962) containing 0.1 μM 2,4-D and 0.3% Gelrite. Two to four weeks later, the shoots obtained were transferred to the same medium devoid of 2,4-D.

For pair-wise statistical comparison of results obtained from different treatments, the parameter-free Fisher's Exact Test (Agresti 1992) was employed.

Flow cytometric analysis

Freshly harvested leaf material of young plants was submerged in 3.5 ml of CyStain UV Ploidy Buffer (Partec, Muenster, Germany) and chopped with a rasor blade. After having passed the suspension through a 30-μm filter tric, at least 5000 isolated nuclei were run through a Ploidy Analyser PA I (Partec, Muenster, Germany) and assessed for their chromatin content according to the instructions of the manufacturer. The allotetraploid reference value was obtained from leaf material of three independent control plants of cv. DZ-01-196.

Results and discussion

Pistil culture

The impact of developmental stage and pretreatment of dissected *E. tef* (Fig. 1a) and *E. mexicana* pistils on enlargement, callus formation and regeneration were investigated. In both species, unpollinated pistils of all tested developmental stages enlarged in culture for a period of about 5 days and normally collapsed afterwards. However, a few of the pistils taken from *E. mexicana* florets at the bi- or tricellular pollen stage or from the tef variety DZ-01-196 at the bi-cellular pollen stage continued to enlarge and eventually formed friable callus. Summarising the results shown in the Tables 1 and 2, six green plants were obtained from

Table 1 Effect of developmental stage on culture of dissected pistils of *E. mexicana* and *E. tef* cv. DZ-01-196

[a]Percentages are given in parentheses

Genotype	*E. mexicana*			E. tef cv. DZ-01-196		
Pistil size	Small	Medium	Large	Small	Medium	Large
Cultured pistils	50	65	71	45	58	50
Enlarged pistils[a]	12 (24.0)	31 (47.7)	67 (94.4)	5 (11.1)	46 (79.3)	40 (80.0)
Callus-forming pistils[a]	0 (0.0)	1 (1.5)	9 (12.7)	0 (0.0)	2 (3.4)	0 (0.0)
Differentiation	–	–	2 green plants	–	Only roots	–

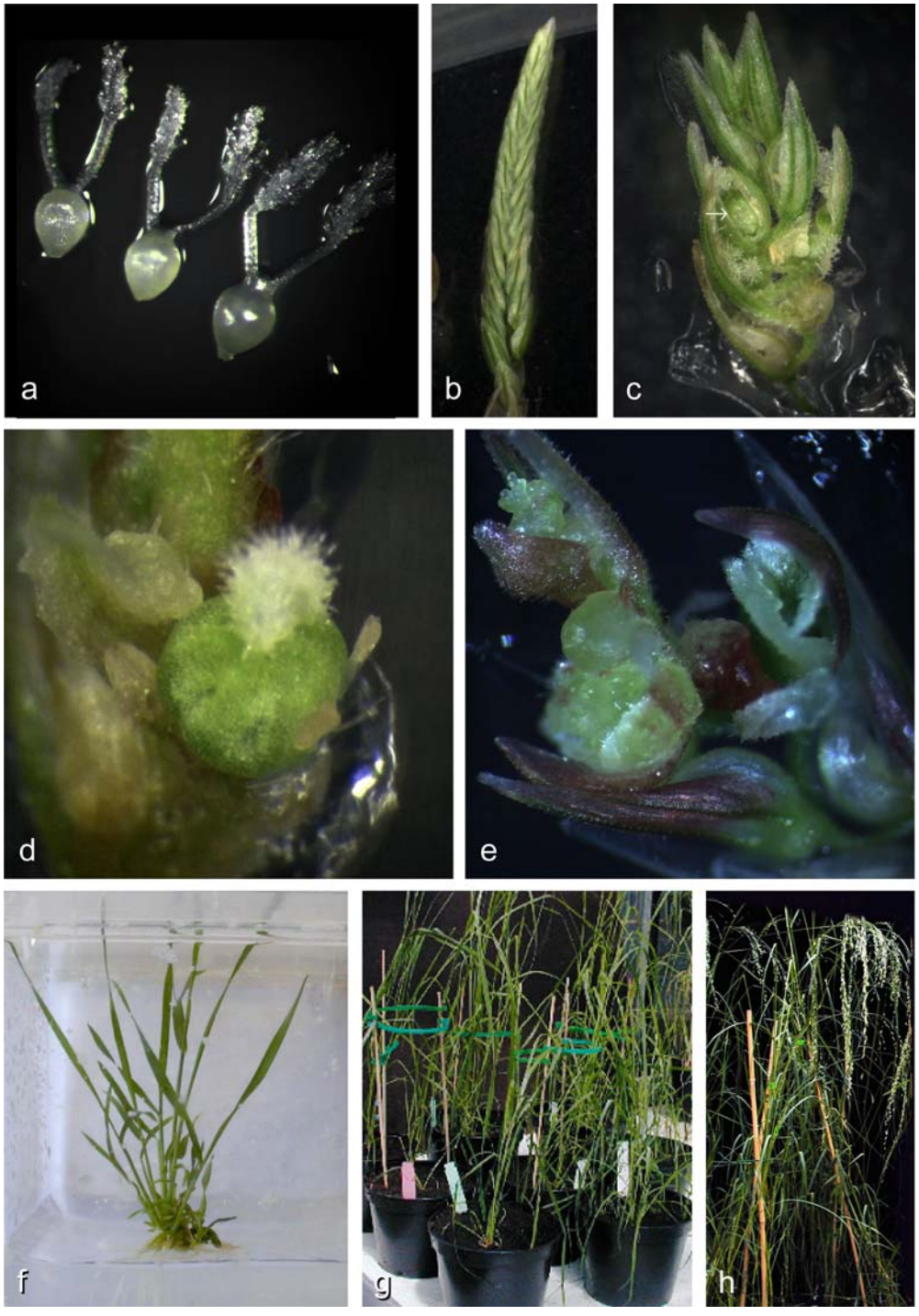

Fig. 1 Gynogenic development of tef cv. DZ-01-196: **a** isolated immature pistils, **b** detached spikelet in culture showing accessory floret formation (note that the normal number of florets per spikelet grown in planta is up to 10), **c** detached spikelet showing ovary enlargement (arrow) after 1 week in culture, **d** enlarged pistil with the lemma removed, **e** embryonic tissue grown out of an enlarged ovary after 3 weeks of panicle segment culture, **f** rooted regenerant **g** regenerants transferred to soil, **h** regenerated plants at flowering

Table 2 Effect of cold pretreatment on culture of dissected pistils of *E. mexicana and E. tef* cv. DZ-01-196

Genotype	*E. mexicana* (large pistils)				DZ-01-196 (medium-sized pistils)			
Pre-incubation of cultures at 4°C	No	1–3 days	4–6 days	7–9 days	No	1–3 days	4–6 days	7–9 days
Cultured pistils	50	61	60	112	61	50	61	112
Enlarged pistils[a]	48 (96.0)	34 (55.7)	58 (96.7)	96 (85.7)	34 (55.7)	15 (30.0)	60 (98.4)	56 (50.0)
Callus-forming pistils[a]	6 (12.0)	4 (6.6)	2 (3.3)	0 (0)	4 (6.6)	2 (4.0)	2 (3.3)	0 (0)
Differentiation	4 green plants	Only roots	Only roots	No	Only roots	No	Only roots	No

[a]Percentages are given in parentheses

121 *E. mexicana* pistils dissected 2–1 days before anthesis, which corresponds to a regeneration efficiency of almost 5%. This result is in line with an earlier report of Castillo and Cistué (1993) on barley who found that the maximum number of haploid plants were produced from ovaries at the tri-cellular pollen stage. Only root formation was observed in the few, merely amorphous calluses in *E. tef*, but no shoot was obtained. Cold pre-incubation of the pistil cultures at 4°C for up to 9 days in *E. tef* and *E. mexicana*, respectively, did not significantly improve gynogenic development (Table 2). Likewise, pre-incubation of the dishes at 32°C for 1 day, culture under light conditions as well as use of liquid medium did not result in improved response of dissected pistils (data not shown).

Spikelet and panicle segment culture

In spikelet and panicle segment cultures, accessory floret development was found to be a common phenomenon (Fig. 1b). While spikelets grown in planta typically carry no more than 10 florets, the number of developed florets of cultured spikelets reached up to 35 with an average of 17 per spikelet. Moreover, some few florets with twin or even triple pistils were observed and individual pistils having three instead of two stigmas or up to seven instead of three anther primordia were found in vitro. Secondary floret growth in spikelet or immature inflorescence cultures were also reported by Brettell et al. (1980) in sorghum, Tefera and Chapman (1992) in tef, Arya et al. (1993) in *Amaranthus paniculatus* and Benkirane et al. (2000) in durum wheat. The development of accessory florets commenced few days after culture initiation and continued until the more basal spikelets attained straw colour.

While in isolated pistil culture, ovary enlargement appeared to be longitudinal, medium-staged pistils of cultured spikelets and panicle segments enlarged rather spherically. The styles were included in this type of pistil expansion and thus appeared to be shortened with still feathery stigmas on top (Fig. 1c and d). The enlarged ovaries were often encircled by a white spongy mass likely deriving from proliferating epidermis. Expanded pistils were up to 15 times larger than those grown in planta. However, the development of the respective anthers was retarded resulting in pale, more or less primordial structures which did not undergo filament elongation (Fig. 1d). In secondary florets however, anthers occasionally attained normal size and colour but microscopic examinations revealed that such anthers did not contain viable pollen.

After about 10 days of culture, embryonic tissue emerged out of the enlarged ovaries at their micropylar end and often entirely overgrew the pistil within the following 2 weeks (Fig. 1e). The mode of embryonic callus formation in spikelet and panicle segment culture appeared to be rather direct than via a distinct intermediate period of complete de-differentiation. Pistil enlargement and gynogenic tissue formation was typically found as of the fourth floret of responding spikelets (Fig. 1c). In spikelets undergoing accessory floret formation, gynogenic development sometimes occured even beyond the 15th floret. After transfer of the entire explants to shoot induction medium (after 10–15 days), the formation of shoots commenced. Upon transfer to regeneration medium, such shoots typically formed roots (Fig. 1f). After transfer of the regenerants to soil, they easily established and underwent normal plant development (Fig. 1g and h). The majority of plants appeared to be fertile and set seed, yet sterile and partially fertile regenerants were also observed. Since tef is a strictly self-pollinating species, the seeds are very likely to derive from self-fertilisation. In turn, self-incompatibility can be largely ruled out as a reason for the sterility observed in some of the regenerated plants.

To determine the optimal developmental time point to initiate gynogenic development in spikelet culture, we compared five developmental stages spanning panicles with early microspores to tri-cellular pollen. Spikelets from panicles which had emerged between 14 and 17 cm out of the flag leaf sheath responded significantly different in gynogenic tissue formation than all other developmental stages (Table 3). A maximum of four florets per spikelet enlarged and formed embryonic tissue when panicles of this stage were used.

Pre-incubation of isolated spikelets or panicle segments at 4°C for various durations showed that gynogenic tissue formation can be improved through cold treatment. In panicle segment cultures, the proportion of spikelets forming gynogenic tissue was significantly increased following pre-incubation for 36 h in comparison with non-pretreated panicles (Table 4). By contrast, Zhou et al. (1986) did not observe an increased gynogenic response in rice panicle culture following cold pretreatment. However, our experiment unambiguously revealed that induction of gynogenic development is more efficient in panicle segments compared to isolated spikelets (Table 4). In rice, gynogenic development was also most efficient when entire florets with intact pistils, stamens and glumes attached to a piece of receptacle was cultured as a unit, while dissected pistils did not respond (Zhou and Yang 1981).

Table 3 Effect of panicle stage on gynogenic tissue formation in detached spikelet culture of *E. tef* cvs. Fesho and DZ-01-196

Panicle emergence (cm)	Cultured spikelets	Gynogenic tissue formation[a]
Fesho		
0–5	170	0 (0.0)
6–9	215	4 (1.9)
10–13	340	14 (4.1)
14–17	420	38 (9.0)*
18–20	540	10 (1.9)
DZ-01-196		
0–5	210	0 (0.0)
6–9	430	19 (4.4)
10–13	660	42 (6.4)
14–17	750	104 (13.9)*
18–20	810	33 (4.1)

[a]Percentages are given in parentheses

*Significantly different to the results obtained using any of the other panicle stages of the respective tef line (Fisher's Exact Test, $p<0.01$)

Table 4 Effect of cold pretreatment on gynogenic tissue formation in detached spikelet and panicle segment culture of *E. tef* cv. DZ-01-196

Pretreatment at 4°C (h)	Cultured spikelets	Gynogenic tissue formation[a]
Spikelet culture		
No	500	4 (0.8)
12	335	3 (0.9)
36	435	5 (1.2)
72	362	2 (0.6)
Panicle segment culture*		
No	840	102 (12.1)
12	830	109 (13.1)
36	960	138** (14.4)
72	794	61 (7.7)

Panicles were harvested at 14–17 cm of emergence

[a]Percentages are given in parentheses

*All results from panicle segment culture were significantly different compared with the respective pretreatments followed by spikelet culture (Fisher's Exact Test, $p<0.01$)

**Significantly different to the results obtained without pretreatment of panicle segments (Fisher's Exact Test, $p<0.01$)

Preliminary experiments had shown that 2,4-D was indispensable for pistil enlargement and gynogenic tissue formation (data not shown). For further optimisation, four different concentrations of 2,4-D (9.2, 18.4, 27.6 and 36.8 μM) were compared across culture of segments from the top, central and basal panicle thirds. This experiment revealed that the central panicle part was significantly more efficient in gynogenic development at all 2,4-D concentrations (Table 5) than basal and top segments. As a consequence, only panicle segments from the central part were used in the following experiments. Among the four levels of 2,4-D concentration tested, gynogenic tissue production was found to be highest at 18.4 μM (39.4%). This concentration lead to a four-fold increase compared to the former standard concentration (9.2 μM). However, at concentrations beyond 18.4 μM induction of gynogenic development decreased significantly revealing that 18.4 μM constitutes an optimum (Table 5). In cultured rice ovaries, concentrations exceeding 2.5 μM of 2-methyl-4-chlorophenoxyacetic acid (MCPA) also lead to decreased induction of gynogenesis (Zhou and Yang 1981; Yang and Zhou 1990).

After optimization of various culture parameters, a final experiment including three *E. tef* cultivars was conducted in which all optima found so far were applied (Table 6). Segments from the central third of panicles which had emerged 14–17 cm from the flag leaf were pretreated for 36 h at 4°C prior to culture on MS medium containing 18.4 μM 2,4-D. In cv. DZ-01-196, out of 1305 spikelets cultured attached to panicle segments 504 pistils formed embryonic tissue. Further subculture on shoot induction and regeneration media resulted in 159 rooted plantlets which were transferred to soil and cultivated in the glasshouse. All plants obtained from any of the donor lines used grew vigorously without albino formation.

There were several indications that the embryonic development observed in spikelet and panicle segment cultures typically did not derive from accidental pollination events, although non-emasculated florets were mainly used. Culture of pollinated spikelets typically resulted in normal seed development in vitro as was shown by Tefera and Chapman (1992) and confirmed in our laboratory (unpublished data). The general amenability of tef for gynogenic development had been shown by the experiments on culture of isolated pistils. Moreover, anthers appeared to cease their development under the culture conditions used (Fig. 1c) and pollen-shedding anthers were never observed inside cultured spikelets. Emasculated spikelets which have been included in some experiments formed embryonic tissue as did non-emasculated ones. Also, the most mature inflorescence stage tested which might be most likely able to form functional pollen in vitro showed substantially less embryonic development than did younger stages. Furthermore, pollination in planta is normally followed by degeneration of the stigmas which was never observed when explants were cultured at any stage prior to anthesis (Fig. 1c and d). As a consequence, pistil enlargement and embryonic tissue formation are very unlikely to be a result of self-pollination under the culture conditions used in this study; however, rare pollination events cannot be entirely ruled out.

A flow-cytometric analysis of 182 plants derived from the experiment shown in Table 6 revealed 5 (di)haploids, 2 triploids, 174 tetraploids and 1 octoploid plant. The flow-cytometric results obtained from a diploid and a tetraploid regenerant along with that of an allotetraploid control tef plant are shown in Fig. 2a–c. In isolated microspore culture of cereals, around 90% of the regenerated plants can be doubled haploids due to spontaneous diploidisation during the culture period (Li and Devaux 2003; Kumlehn et al. 2006). However, spontaneus genome doubling is a rather rare event in most gynogenesis-based regeneration systems. Especially in cereals, gynogenesis has been reported not to result in spontaneous genome doubling (e.g. Castillo and Cistué 1993). As yet, there are only a few published data

Table 5 Effect of panicle segment position and 2,4-D concentration on formation of gynogenic tissue in panicle segment culture of cv. DZ-01-196

2,4-D (μM)	Panicle segment position					
	Basal		Central		Top	
	Cultured spikelets	Gynogenic tissue formation[a]	Cultured spikelets	Gynogenic tissue formation[a]	Cultured spikelets	Gynogenic tissue formation[a]
9.2	217	17 (7.8)	436	47 (10.8)	127	7 (5.5)
18.4	251	41 (16.3)*	355	140 (39.4)**	333	25 (7.5)
27.6	235	16 (6.8)	388	36 (9.2)	197	11 (5.6)
36.8	268	7 (2.6)	449	33 (7.4)	188	7 (3.7)

Panicles were harvested at 14–17 cm of emergence and no temperature pretreatment was applied

[a]Percentages are given in parentheses

*Significantly different to the results obtained following culture of basal segments with any other 2,4-D concentration (Fisher's Exact Test, $p<0.05$)

**Significantly different to the results obtained following culture of central segments with any other 2,4-D concentration as well as following culture of basal or top segments using 18.4 μM 2,4-D (Fisher's Exact Test, $p<0.001$)

showing remarkable spontaneus diploidisation in the context of gynogenesis, e.g. in sugar beet (Lux et al. 1990) and onion (Michalik et al. 1997). The above described circumstances of the method established in this study along with the finding that haploid plants are among the regenerants obtained suggests that the tetraploid plants identified are likely to be the result of spontaneous genome doubling; however, the cellular origin as well as the homozygous nature of these plants have yet to be elucidated. Since tef is an inbreeding species, its inherent homozygosity makes it difficult, if not impossible, to compellingly show whether the tetraploid regenerants are true doubled haploids. A molecular marker system that provides the resolution required to enable visualisation of segregating alleles within a single tef line is as yet not available. However, the first results on the development of tef AFLPs are fairly promising (Bai et al. 1999).

Perspectives

The implementation of doubled haploid technology has tremendously accelerated the breeding progress, e.g. in barley, wheat, rice, maize, rape, potato, sugar beet and onion. Consequently, tef-breeding programs would also greatly profit from the opportunity to produce doubled haploids. However, the particular cellular origin and the homozygous nature of the tetraploid plants obtained by the method presented here yet awaits experimental elucidation.

Table 6 Gynogenic tissue formation and plant regeneration using an optimised protocol for panicle segment culture

	Cultured spikelets	Gynogenic tissue formation[a]	Regenerated plants[a]
E. mexicana	550	0 (0.0)	0 (0.0)
E. tef cv. Fesho	1210	392 (32.4)	100 (8.3)
E. tef cv. CR-37	800	88 (11.0)	9 (1.1)
E. tef cv. DZ-01-196	1305	504 (38.6)	159 (12.2)

Panicles were harvested at 14–17 cm of emergence and central segments pretreated at 4°C for 36 h. The induction medium was supplemented with 18.4 μM 2,4-D

[a]Percentages are given in parentheses

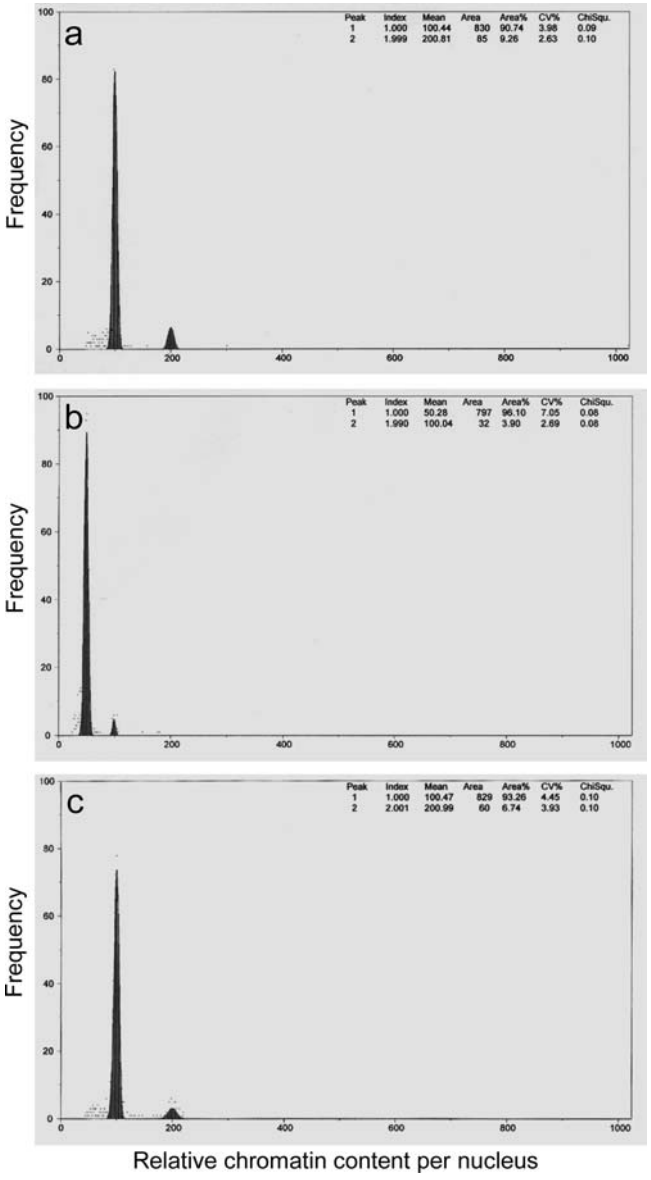

Fig. 2 Flow-cytometric ploidy analysis of regenerants obtained from panicle segment culture of tef cv. DZ-01-196: **a** control plant showing the allotetraploid genome size of tef, **b** (di)haploid regenerant, **c** tetraploid regenerant

Also, the amenability of haploid tef plants to chemically induced genome doubling has to be figured out and a respective method established. In addition to the potential employment for doubled haploid formation, the method established here may encourage further work, e.g. towards the development of a embryo rescue technique for very early stages of hybrid embryos showing post-zygotic incompatibility. Such a method would greatly improve the opportunities for hybridisation approaches to broaden the available germplasm potentially contributing to future tef improvement, e.g. to introduce valuable traits such as disease resistances or lodging and drought tolerances from the numerous wild relatives of tef.

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