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SCHOOL OF GRADUATE STUDIES
ADDIS ABABA, ETHIOPIA

AND

UNIVERSITÄT HAMBURG, INSTITÜT FÜR BIOZENTRUM, KLEIN FLOTTBEK ÜND BOTANISCHER GARTEN (APPLIED MOLECULAR BIOLOGY OF PLANTS) HAMBURG, GERMANY

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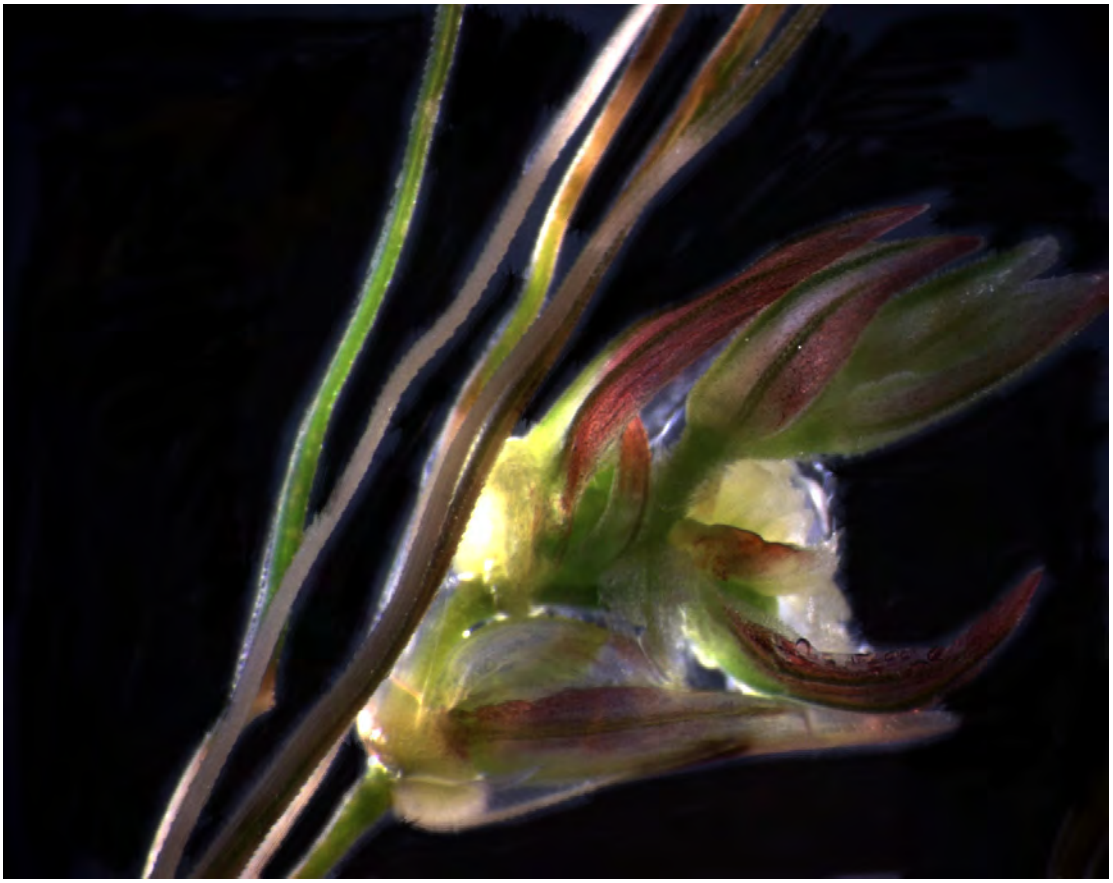

LIKYELESH GUGSA TUFFER

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A Ph.D Thesis Submitted to the School of Graduate Studies, Addis Ababa University, in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Biology

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JUNE 2005

Dedication

This thesis is dedicated to my brother, Tilahun Gugsa and my nephew, Yosef Girma, who passed away during the period of my study

ACKNOWLEDGMENT

I express my sincere gratitude and indebtedness to my supervisor Professor Dr. Horst Lörz for accepting me as a PhD student as well as providing facilities in his laboratory, Institute of Biozentrum, Klein Flottbek and Botanischer Gartern AMPII, University of Hamburg, where I worked my thesis project. I am grateful to him for his unlimited encouragement throughout my study period and his scholar responsibility and kind support during the need of official communication regarding my hard times which are unforgettable. Besides, his support and collaboration to facilitate working condition and very kind understanding was a key factor during the study period.

I am also highly grateful to Dr. Jochen Kumlehn, Head, Plant Reproduction Biology, IPK, Gatersleben, Germany who started to be my official co-advisor when he was in Hamburg University. However, after two months of supervision, he left for IPK. Although he was away from Hamburg during the critical time of my study, he kept in touch with me and contributed a lot to my successful results. Almost all the culture protocols developed for tef in this study using K99 medium were kindly provided by him. Moreover, I am very grateful to him for allowing me for four months stay in his laboratory at the IPK to study on tef transformation. I am deeply indebted for his valuable guidance and cordial attitude and inspiration throughout my study and also for his critical comments and suggestions.

I wish to express my special thanks to my advisor, Professor Endashaw Bekele for accepting me as a PhD student and his recommendations to the DAAD and his helpful comments on my PhD proposal. I also deeply thank him for his encouragement and facilitating my registration at AAU. Besides, I heartedly thank him for his critical, correction, discussions and criticisms during the final thesis write up. I also thanks all colleagues of the AMPII, Hamburg who made my stay in the laboratory very pleasant. Special thanks are due to Mrs. R. Smith and Dr. Ana Satival for their special love and visit during my stay in the hospital and at Altewache Hotel for some months. My thanks also goes to Dr. Manfred Gehrtz, for his valuable suggestions during the study and helping in photographing, Mrs. Marlis Nissen, for her unfailing assistance in the laboratory, Mrs.U. Loffler, Dr. P. Schwarz and Mrs. Ingrid Otto for the flow cytometry analysis.

I thank deeply Mrs. Otto for helping and taking care of the transformation experiment including the Southern blot analysis of the successive generations of the transformed plants of tef after I left Germany. My thanks are also extended to all IPK, PRB group specially to those who showed me the particle gun gene delivery and agrobacterium mediated transformation system. Besides, the Gardeners of Hamburg University and IPK are acknowledged for managing the plants in the respective greenhouses.

I am deeply grateful to Deutscher Akademischer Austauschdienst (DAAD) for the three years fellowship to study my PhD in Germany and the management of the Ethiopian Agricultural Research Organization (EARO) for granting me a study leave to pursue my PhD degree

I am thankful to the past and present Heads of the department of biology, Dr Kifle Dagne, Dr. Dawit Abate respectively, and express my special thanks to the Department academic committee members, for facilitating the final part of my study and timely completion of my defense at the AAU. I am also very thankful to Prof. Dr. Legesse Negash for his precious time and kindness to use his microscope for photographing at AAU. My thanks is also extended to Dr. Ashok K. Sarial, Associate Professor (Genetics) for his valuable advice during the preparation of my thesis and seminar paper. I am also grateful for his understanding, and kindness to allow me to use his office for my work.

My thanks also extended to IBC and Holetta Research Center management and colleagues of tef section especially to Mrs. Desta Birhanu, Mr. Dawit Girma, Mr. Gezahegne Getaneh, Mrs Woynishet W/Semayat and others for taking care of my independent study course materials at the lath house and for their kind help to provide computer facilities. I also thank Mr. Tadesse Yimer for his help during the final print of the thesis. My thanks also forwarded to other many friends like Mrs. Enanu Tekle for their encouragement and help.

My very special thanks go to the Hamburg Altona Krankenhous and Reha Zentrüm doctors, nurses and specialists who took care of me in the hospital due to the accident happened during the earliest period of this study.

I am deeply thankful to all my friends from the Ethiopian Christian believers church (Bethel church, Hamburg) who showed their hospitality, love and prayer during the hardest period in my life. My Thanks are extended to the Haak family for their unfailing love, help and care during
my three years stay in their rented house. Especially the motherly love of Mrs. Haak was very helpful for the success of my study and for my healing from many of my hard times, for example during my deep hurt specially during the accident and the lose of my nephew Yosef Girma.

I cherish the love and encouragement of my mother Mrs Adanech Habte and my father Grazmach. Gugsa Tufer, my brothers Mr. Girma Gugsa, Mr. Baddeg Gugsa, and his wife Mrs. Senait Kebede, Mr. Amare Gugsa, Mr Genene Gugsa, Mr. Tewodros Gugsa and my sisters, Sister Felekech Bulbula, Mrs. Yewbnesh Gugsa, Mrs. Netsanet Gugsa, and all my sister in-laws and brother in-laws and to all my nephews and nieces. I specially thank Mrs. Yewbnesh for frequent visit of my children and taking responsibilities especially taking care of our small daughter. I also deeply thank for all my baby sitters, especially to Trunesh Petros who took care of our house during my absence and until the completion of this study at the AAU.

Finally my beloved ones, my husband Dr. Yaynu Hiskias, my sons Yonathan and Amanuel Yaynu and my little daughter Eden Yaynu are most thankful due to their deep affection and patience to stay alone during the period of my study abroad. I specially thank my husband for taking care of me during my study at the AAU and his encouragement to achieve my goal from the beginning of my higher education career.

Last but not least I give praise and honor to my heavenly Father the Almighty Lord for his unfailing and unconditional love, absolute healing and taking care of me and my family, leading the way, giving the capacity, tolerance and ability to carry out and accomplish this heavy and pioneer work being away from family for three and half years. Dear my God, all the success of this study is due to your kind help and guidance I followed your word, which says **seek and you will find it** and I sought in your name and I found successful results of my study objectives due to you. I praise your name for all my life.

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ABSTRACT

The four objectives of this PhD study were to develop protocols for embryo rescue technique, efficient plant regeneration system from immature embryos, *in vitro* haplodization either through androgenesis or gynogenesis and transformation in *Eragrostis* tef. The major study materials include were three tef varieties, improved, DZ-01-196 and DZ-CR-37, a farmers variety, Fesho and the wild, relatives *E mexicana, .E, pilosa* and *E.papposa*. For embryo rescue study, both zygotic embryos and ovaries at early stage of self pollination were excised and cultured in the medium. More than 90 % rescue was recorded for tef varieties from both explants. Similar results were obtained from some wild species from embryo culture. DZ-01-196 performed better than DZ -CR-37 and Fesho in both explant types.A highly efficient *in vitro* culture system was also developed using immature embryos of tef. Efficiency was examined by the embryogenic calli formation, plant regeneration and number of shoots per explant obtained. 100 % somatic embryo formation, 98.2% plant regeneration as well as maximum number 565 plantlets per single explant were obtained from variety DZ-01-196. This highly efficient technique developed here will be very useful for further studies like transformation which required high plant regeneration system. In androgenesis study factors influencing androgenic response were investigated. 0.15 % of calli were induced from the cultured microspores and only one albino plant was obtained. This result showed that tef is recalcitrant to androgenesis. Therefore, *in vitro* gynogenesis was attempted. Out of 14234 cultured unpollinated pistils, 2035 embryonic tissues and 13.4 %. regeneration were obtained The flow cytometry analysis of these plants revealed 5 haploids (di-haploids), 2, aneuploids, 172, tetraploids and 1 octoploid plant. Haploid and variant polyploidy plant production in tef is for the first time. For tef transformation study, genes for selectable marker and reporter genes were used and 4 transformed plants from biolistic and 1 transgenic plant from agrobacterium method survived the repeated sub culturing on selection media. Transgenic plants, recovered after 4-6 weeks from the time of gene transfer were subjected to PCR and southern blot analysis. The result revealed from T1 plants stable transformation from both transformation system. This protocol will contribute for the improvement of the crop in the near future.

Key words: *Eragrostis tef*, ovary, immature embryo, rescue, pistil, microspores, haplodization, androgenesis, embryonic tissue, gynogenesis, biolistic and agro-bacterium transformation.

Chapter 1

General Introduction

1.1 The plant

Tef, (*Eragrostis tef* (Zucc.) Trotter is commonly known as Tef, Teff and T'ef. It is a C4 plant (Kebede et al., 1989), having an anatomical characteristic that is intermediate between a tropical and temperate grass (Stallknecht et al., 1993). Tef is a fine stemmed tufted annual grass and produces small seeds (1000 weigh 0.3 to 0.4 g). The plant has the appearance of a bunch grass having large crown and many tillers. The roots are shallow and develop a massive fibrous rooting system. The inflorescence is mainly open panicle but range from very loose to very compact. Plant height of tef varies based on cultivar type and growing conditions.

1.2 The crop

The production and use of tef in Ethiopia can be traced back to about 3500 BC (Mengesha, 1965). However, a precise evidence for this is not yet worked out. In contrast to amaranth, which was utilized by early civilizations throughout the world, tef production and uses have been primarily restricted to Ethiopia (Anon, 1894) and later may be after early 1919 continued to British colonies including Australia (Anon 1894). Ethiopia is both the origin and center of diversity for tef (Costanza et al., 1979). At present since tef has very low or little gluten; its demand is expanding to Europe and North America as a low gluten crop.

Tef is an indigenous and important food and feed crop of Ethiopia and occupies a premier position in area coverage among the various food crops cultivated in Ethiopia (Fig.1). It is grown annually almost exclusively on about 2 million hectares (Central Statistical Authority, 2004) of land (Table 1), there by covering 30 % (largest) of the total acreage of cereals and about 26% of the whole area cultivated to annual field crops. Area coverage is expanding from year to year and the maximum reached so far was in the 1996/97 cropping season which was 2.23 million hectare and the highest production of tef was obtained in the same year (Table 1) (CSA, 2004).

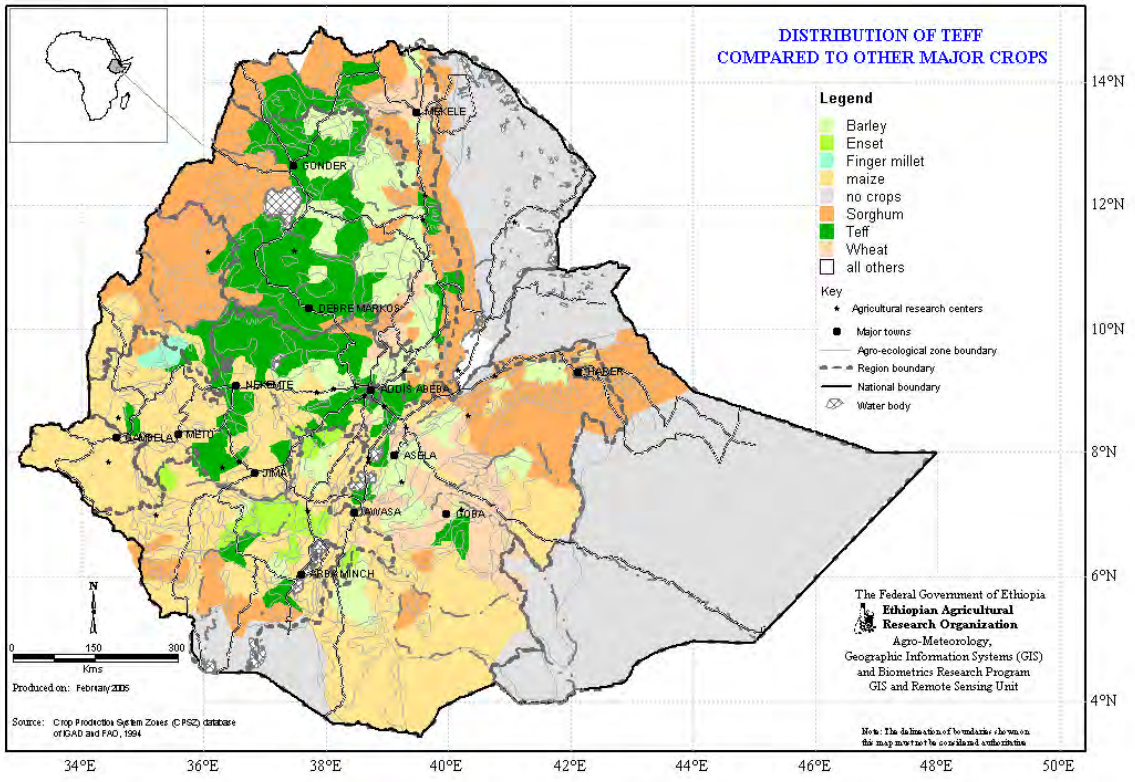


Fig 1. Area coverage of tef compared to other cereals crops grown in Ethiopia

Table 1. Area, production and yield of tef from the year 1993/94-2003/04 (main growing season)

Year ⟶	1993/94	1994/95	1995/96	1996/97	1997/98	98/99	1999/2000	2000/01	2001/02	2002/03	2003/04	**Mean**
Area, mill. ha	1.4	1.9	2.16	2.23	1.8	2.1	2.12	2.2	1.8	1.9	1.99	1.96
Production in (mil. quinta	12,6	18.6	17.93	20.4	13.3	16.4	17.17	17.4	16.3	14.2	16.77	16.45
Yield /ha	8.8	9.8	8.3	9.1	7.5	7.8	8.09	7.96	8.95	7.4	8.43	8.37

Tef is produced in seven regions of the eighteen agro ecological regions of the country at varying degrees. The Amhara and Oromya regions have the largest acreage followed by Southern Nations Nationalities and Peoples Region (SNNPR) and Tigray. There is no report on the cultivation of the crop in Somalia, Harare and Dire Dawa administrative regions. However, 3

zones in Beneshangule (Metekel, Asossa and Klmashi) and 3 zones in Afar regions have started producing tef (Central Statistics Authority, 2004).

Tef ranks first in importance amongst the food crops grown in the country, with an annual production of 1.6 Metric tonnes. According to Ketema, (1993) there are five main reasons for the popularity of tef. Its importance is based firstly on its high demand by the consumers. Secondly, its agronomic versatility and reliability, even under adverse condition, which suit it well to a country of contrasting and unpredictable environment, where drought, water logging, pests and disease cause recurrent famine (Ketema, 1993). Thirdly, besides its value for human grain, tef straw is equally important for livestock forage. Fourthly, tef straw reinforces mud in the construction of local buildings. Fifthly, tef can be produced in a relatively short growing season and will produce both grain for human and fodder for cattle. Therefore, tef assumes a peculiar position in the Ethiopian agricultural systems.

The FAO kilo caloric equivalence for 1 kg of grain indicated that tef's, kilocalorie, 3670 is higher than all other cereals and even better than most pulses except peanut, 5670 (Nutrition Technical Assistance (FAO, FANTA), 2004). Therefore, multifarious merits, agronomic and ecological versatility, biological features of drought and waterlogging tolerance, straw quality comparable to native hay and economic supremacy over other cereals and lastly, the relative imperishable of "Enjera" contributed to the crop's popularity in Ethiopia both for the grower and consumer. In the face of population increase coupled with solid consumer demand, the price of tef is escalating making it the most expensive cereal in the country. This will continue for some time to come unless the *status quo* in tef grain production is significantly altered. High research investment will help raise the share of tef in the cereal requirement of the nation. And this in turn requires concerted effort from all respective professionals.

1.2.1 Economic importance:

Tirfe and Mekonen (1995) accounted the persistent popularity of tef as staple diet in Ethiopia to five major reasons: (1) in the highlands certain areas are more suitable for its production than other alternative crops, (2) less susceptibility to weevil attack, (3) little price fluctuation in tef compared with other crops, demand in most urban areas of the nation and even in the markets of neighbouring

countries, namely Eritrea, Djibouti and the Sudan, (4) tef enjera has more shelf life than similar food made from other crops, and (5) high extraction rate when converted to flour-and there is less waste in processing tef to various forms of food. Furthermore, attempts to displace tef from its premier place in the national agricultural system failed mainly due to its ecological versatility, its usefulness in marginal conditions and its economic supremacy over the other cereals (Ketema, personal communication).

Tirfe and Mekonnen (1995) analysed the prospects for grain market and reported the existence of high demand for tef and pulses in the urban areas. Table 2 illustrates the income generated from tef grain export mainly for immigrants of Ethiopia living abroad. Tef supply was also found to influence the price of other cereals such as maize. When the supply of tef is good, the price of maize was observed to fall and the year tef supply is unfavourable, the demand for maize rises. This variation in price is likely to encourage farmers to allocate more land for the production of tef and wherever the climate is conducive to grow both crops. This instability of price from one year to the other influences farmers to plant tef despite the fact that yield per hectare for maize is greater than tef.

Table 2. Amount of tef grain exported and value (Birr) obtained in 1997/98 and 1998/1999

Year	1997/1998	1998/1999
Quantity exported in tonnes	1797.68	1840.28
Value (mill. birr)	8.87	14.57

Source**:** Customs Authority, Addis Zemen (Ethiopian News Agency), Tikmit 13, 1992 EC

Being an almost exclusively small holder crop, tef is chosen by farmers for use in a range of ecological zones from cold plateaux at up to 3000 masl often on water logged prone vertisols, down to hot dry regions below 1500 masl (Cheverton, 1985). In dry conditions its short growing period and relative resistance to drought permit a harvest even after crops such as maize or sorghum have failed. In water logged conditions its growth is stunted, but yield is depressed less than other crops such as wheat or barley. In terms of both national and local economy, tef is uniquely important over much of the upland Ethiopia.

1.2.2 Grain quality of tef: The principal use of tef grain for human food is the flattened bread (enjera) that resembles a pancake. Grain quality at present is restricted only to colour. A good quality tef enjera has a smooth texture, slightly sour but pleasant flavour and is pliable (Wondimu and Tekabe, 2000). The grain colour covers ranges of colours like white, brown and very deep reddish brown (Ebba, 1975). White enjera is considered as the best quality and fetches high price in the market.

1.2.2.1 Nutritional quality of tef: The grain protein content of tef (10-12 %) is similar to other cereals (Bekele and Lester, 1981). Jansen et al., (1962) reported that tef has an excellent balance of essential amino acids. Besides providing protein and calories, tef is a high source of minerals, such as of calcium, phosphorous, copper, aluminium, barium, thiamine and particularly iron (Mengesha, 1965). The content of iron in tef is higher than that of other cereals (Mengesha, 1965). Besides, Ebba, (1969) reported that non-tef consumers have a lower level of hemoglobins and therefore are prone to develop hookworm anaemia if infested. In addition, according to the same author malaria is frequently found in the groups with lower hemoglobins levels. The high iron content of tef seed has been refuted by some authors, however, the lack of anaemia in Ethiopia is considered by many to be due to the available iron from enjera (Mamo and Parsons, 1987).

Research studies have shown that if the fermentation process is prolonged to produce the sour type of enjera, essential nutrients particularly amino acids such as lysine are lost in the liquid, which is removed from the dough. The nutrient loss can be reduced if the fermentation process is shortened but then the result is a sweet type of enjera, which has a shorter shelf life. Traditionally tef is consumed with 'Wot', a soup made of meat, or ground pulses like lentil, faba bean, field pea, broad bean and chickpea. These supplement lysine and provide a better balanced diet. Furthermore, tef has low gluten, which make it highly demanded crop for coeliac disease patients, who are sensitive (allergic) to gluten. Due to this the grain and flour of tef are found in the health food market of United States, United Kingdom, Germany, Netherlands and others. Recently tef is also used to make pasta and macaroni in Ethiopia. Besides, more than 15 other recipes can be prepared from tef seeds and flour (Ketema, 1993).

In general no variety is yet identified for its high quality (nutritionally) except for seed color and enjera texture where variety DZ-01-196 appeared to be the first rank followed by variety DZ-

CR-37. According to the preliminary study, variety DZ-CR-37 has high water absorbing capacity, thus yielding higher enjera per kg of tef flour (Holetta progress report, 2000).

1.2.2.2 Essential amino acids and proteins in tef: Jansen et al. (1962), Mengesha (1965), Mulugeta (1978), Areda (1990) and Bekele (1995a & b) have studied the essential amino acids and proteins in tef using various number of tef genotypes. They reported that the protein level (11 %) of tef grain is similar to other cereal grains especially of bread wheat. The major fractional composition of tef seed storage protein according to Mulugeta (1978) are glutelins (44.6 %) and albumin (36.6 %) followed by prolamin (11.8%) and globulin (6.7%). However, Bekele (1995a) reported albumin, glutelins, and globulins as the major fractions in their order of importance. Bekele (1995a) stated additionally that the protein fractions of tef vary from genotype to genotype. According to Jansen et al. (1962) and Areda (1990), the amino acid content of tef is in general is excellent and lysine content in particular higher than that of all cereals, except rice and oat. However, Bekele (1995b) reported that lysine, arginine and threonine content of tef are lower than most cereals including winter and spring wheat, spring barley, winter rye and spring oat. Since the genotypes used by these authors were different, the variation reported in the content of lycine is as expected. According to Bekele (1995b), therefore, lysine is limiting in most cereals and there is a need to identify tef genotypes with high lysine and balanced amino acid composition. According to Ketema (1993), supplementation of tef flour by few seeds of rousted and grinded fenugreek (*Trigonella foenugrecum*) (a common practice of the local women) improved the quality of tef enjera in terms of nutrition, taste and baking quality.

1.2.3 The Origin of Tef: *Eragrostis* is a large genus, which comprises about 350 species among which only tef is cultivated as a human crop. About 50 % of the 350 species are native to Africa (Cufodontis, 1974). According to Vavilov (1951)**,** Ethiopia is both the center of origin and diversity for tef. Although, tef has been cultivated for many thousand years, the time and the location of its first cultivations and utilization is not exactly known. Many authors shared the opinion that tef is originated from the wild (*E .pilosa)* L. Beauv) (Braun 1941, cited by Ebba 1975). The characters of *E. pilosa* and *E. tef* are in many respects so similar that it is sometimes claimed that tef should be considered as a subspecies or a variety of *E.pilosa* rather than a separate species (Braun, 1941 and 1948 cited by Ebba 1975). Bekele and Lester (1981)

suggested that *E.pilosa, E aethiopica, E. barrelier* and possibly *E. curvula* and *E. cilianensis* might have been involved in the evolution of tef. Chiovenda (1912) in Ebba (1975), assumed that tef may have originated from *E. aethiopica*. Based on morphological traits, cytological evidence and biochemical markers several wild species identified to be close relatives or progenitors of the present day tef. On morphological grounds Jones et al. (1978) identified nine species that are believed to be close relatives. These are *E.aethiopica, E. barrelieri, E. cilianensis, E. minor, E. pilosa, E. biclor, E. mexicana E. hetromera* and *E. papposa*. According to these authors, *E.pilosa* and *E. cillianensis* complex are the closest to tef. Among which *E.pilosa* is thought to be the closest relative by morphological observation. Besides, *E.pilosa* is widely distributed in Ethiopia and is sexually compatible with tef (Gugsa et al., 1999), and may be useful sources of genes for tef improvement.

The identification of the tef diploid progenitors, based on genome analysis and through the use of biochemical or DNA-based markers, is useful. If the two diploid progenitors are known, there could be an avenue to re-synthesize tef and create new variability including for lodging resistance. According to the recent molecular analysis using Amplified Fragment Length Polymorphism (Ayele et al., 1999; Bai, et. al., 1999) showed low level of polymorphism exhibited between *E.tef* and *E.pilosa*, thus indicating the close relationship between them.

1.2.4 Production and research constraints

1.2.4.1 Genetic: The first and foremost technical factor limiting tef grain yields is a genetic one. Most of the national production of tef grain comes from unimproved land races. These are relatively low yielding genotypes. The national average yield of tef is 0.91 tons/ ha (Ketema, 1993). However, in current years this has a trend of further reduction to 0.8 tons/ ha (Table 1). Besides, economically significant yield is lost due to lodging (Ketema, 1983; Cheverton et al., 1992). Basic knowledge of the crop is severely limited and this has often slowed progress in genetic improvement. In addition, the variation among the improved varieties in terms of yield is not highly significant from one another except their various suitable adaptation areas. Only 5 varieties out of 15 are obtained from the crossing program and the rest are selected from the land races. This is mainly because hybridization is difficult, slow and tedious.

Interspecific and wide crossing could increase the variability of the genotypes which were not detected in nature. However, the success of achieving hybrids even with the close relatives of tef has been difficult.

The other serious problem in tef production is lodging. Lodging can be induced by internal or external factors or by interaction between the two (Ketema, 1983). Lodging at current production condition is also caused by ample amount of nitrogen fertilizer (Urea) application. Other factors contributed to lodging are wind, heavy rain, fungal damage failure to the root system, weakness of the straw, heavy rates of seedling, lack of phosphorous, lack of potassium, and an insufficient amount of strengthen sclerenchyma tissue in the culms (Hamilton, 1951 in Ketema, 1993). Moreover, lodging is most likely to occur when tillers have a high center of gravity and a heavy, high yielding panicle (Cheverton et al., 1992). Tef plant is so weak in nature and can not withstand strong wind, heavy rain and high vegetative growth also made tef to lodge. Lodging occurs before heading (translucent) has no effect. However, lodging occurs after heading causes permanent lodging such as bend, break and root lodging and among which bend lodging in general is economically important one and cause a yield loses of 11-25 % (Ketema, 1983). No lodging resistant variety is yet identified among the tef germplasm.

1.2.4.2 Pest, fertility and management problems: Grass weeds and rust (*Uromyces eragrostidea*) are economically important and deserve investigation (Bekele, 1985). Besides, Helminthosporium leaf spot disease is the major problem in many parts of tef growing area especially around Bako and Western Welega (Bekele, 1985). Furthermore, insects such as grasshoppers, cricket and several others cause damage to seedlings of the crop in many tef production areas. Besides, poor management and no or little application of fertilizer by the farmers contributed to its low productivity.

1.2.5 Tef breeding and genetic research

1.2.5.1 Historical development: The period in the late 1950's marked the beginning of tef improvement research at Jimma Agricultural Technical School and later moved to Debre Zeit. Selection of lines from land races was the first attempt made to improve tef. In the years that followed the quest for a hybridization technique continued for several years without success. In 1972 scientists, briefly, resorted to mutagenesis as a means of creating additional genetic variability.

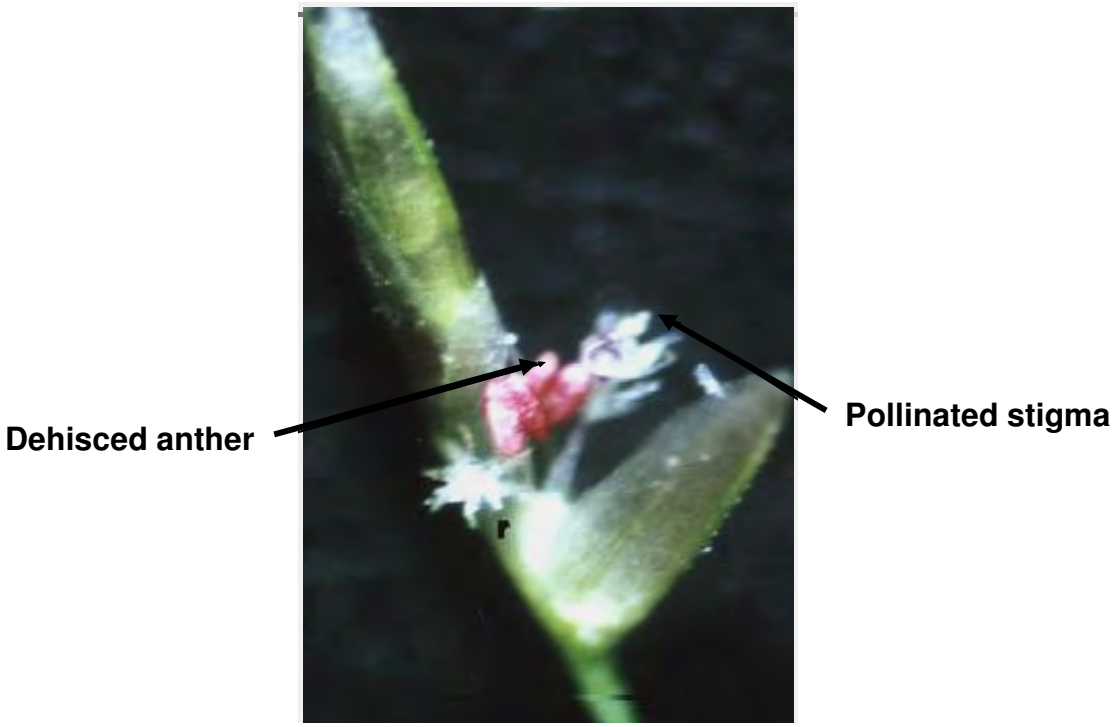
The problem of artificial hybridization in tef terminated with the discovery of the pollination habit of tef two years later in 1974 (Berhe, 1975) and a crossing program marking the third period of tef breeding was realized. Latter on, the governments of Ethiopia recognized the contributions of tef to the national agriculture and food security and accorded a priority status for research.

1.2.5.2 Breeding objectives: So far, breeding program focused on high grain yield, wide adaptation and lodging resistance. Attempts were therefore, made to select for better standing ability as a subsidiary trait to higher grain yield. Cheverton et al., 1990 and his group worked out the mathematical model of the mechanics of lodging resistant, and concluded that lodging resistance can be achieved with plants whose culms are especially thick, and with short heights. However, although much of the breeding work is focused in achieving lodging resistant genotypes, no particular genotypes are detected for its lodging resistant gene in tef. The wild species are considered to be possible donors of this important trait. Breeding for various production zones with varying length of growing season was also done. Breeding for quality, diseases and insect resistance are not yet targeted. Although tef straw is a valuable product, it is primarily grain yield, which farmers identify as their desired objectives. Grain quality is considered in terms of grain colour at present. Baking quality and other aspects are not yet considered seriously.

1.2.6 Conventional breeding

1.2.6.1 Selection and inter varietal crossing (intraspecific crossing): **Tef research is relatively recent. In 1987, one World Bank document judged tef research as being run on a "care-and-maintenance" basis. Decades ago tef was where bread wheat was in 1880, maize in 1910 and sorghum in 1930; these major cereals received thorough scientific investigation to be raised to their present level of productivity (Jones, personal communication). The previous state of global food owes due to all multifaceted scientific effort invested into these crops. Tef breeding program improved varieties of tef with increased grain yield. Interspecific and wide crossing methods also didn't contribute to the diversity of the crop due to the failure of hybrid seeds production.**

1.2.6.2 Crossing technique of tef and its floral biology: Tef floret is hermaphrodite with three stamens and a pistil with two stigmas. For many years tef was believed to be cliestogamous, (flowers do not open for self pollination) (Mengesha and Guard, 1966). However, Berhe, in 1975 discovered that tef flowers open early at the day break. Then he developed a technique for hybridization of tef for the first time. However, crossing of tef is very cumbersome and time consuming. The main obstacle of tef crossing attributed to its unique pollination habit and size of the flower. The flower of tef is very small, on the average 1 millimetre x 2 millimetres (Fig. 2) and it requires a surgical emasculation using needles and fine forceps and pollination with the aid of a low power stereomicroscope at a magnification of X30-X50.



Source: Likvelesh Gugsa.

Fig. 2 Opened floret of tef at anthesis, showing pollinated stigma and dehisced anthers

Besides, flower opening and self-pollination occurs early at the day break, around 6:00-7:00 a.m. Moreover, once the flowers opened, pollen sheds within 30-40 minutes. Therefore, no fresh supply of pollen will be found for hand pollination (Ketema, 1983). Emasculation is essential in tef crossing since there is no male sterility trait found in nature or artificially. According to Ketema, 1983, treatment of inflorescences with hot water may induce sterility. The highest temperature, 48°C, produced male sterility with high exposure. Timing was also critical because treatments for three or more minutes produced female sterility. 44°C gave the most satisfactory results (a high degree of male sterility) after nine minutes, but no effect on female fertility was observed. His work then included hot water treatment of flowers to induce male sterility without disturbing the female organ and he reported that 48°C induced male sterility if treated for three minutes. Extended treatment beyond 48°C induced female sterility.

The improvement of hybridization technique continued by attempting selective male gametocyte such as: Ethrel E. (Ketema, 1983) and Ethephon (Berhe and Miller, 1978; Assefa, 1991) in order

to kill the male gametes so that the tedious emasculation could be avoided. However, these attempts were not successful as expected due to the toxicity of the chemical to the female organ and the difficulty in pollination since the unfunctional pollen still dehisce and covers the stigma.

1.2.6.3 Intraspecific crosses: Wild species are known to be the sources of useful genes such as resistance or tolerance to diseases, pests and abiotic factors (Monti et al., 1992). However, attempts to develop an improved variety by interspecific crosses repeatedly failed in *Eragrostis* may be due to embryo abortion and incompatibility. To overcome abortion problem embryo rescue method need to be developed.

1.2.6.4 Mutation breeding: Mutation breeding in tef started in 1972 with the objective of creating additional genetic variability especially to obtain lodging resistant genotype. Several chemical and physical mutagens have been used to induce mutation in tef to initiate or identify lodging resistant and create dwarf tef lines. However, so far no genotype with positive mutation is identified.

1.2.7 Released varieties:

Conventional breeding and selection programs contributed for the release of 15 varieties (Table 3). These varieties have specific and wide adaptation. Of the fifteen improved varieties only five were obtained from the inter-varietals crossing program.

In water logging conditions its growth is stunted, but yield is depressed less than other crops such as wheat or barley. In terms of both national and local economies, tef is uniquely important over much of upland Ethiopia.

Table 3. The yield performance, adaptation and some agronomic traits of the released tef varieties on farmers and experimental fields

Variety	Year of Release	Plant Height (cm)	Seed Colour	Days to Maturity	Grain Yield, q/ha		Altitude (m)	Seasonal Rainfall (mm)	Region
					Research Field	Farmers Field			
DZ-01-354	1970	53-115	Pale white	85-130	24-32	17-22	1600-2400	300-700	Mid to high altitude
DZ-01-99	1970	53-100	Brown	85-130	24-30	17-22	1600-2400	300-700	Mid to high altitude
DZ-01-196	1970	50-117	Very white	90-130	24-30	14-16	1800-2400	300-700	East Shewa
DZ-01-787	1978	50-110	White	125-140	24-30	17-22	1800-2500	400-700	High altitude(water logged)
DZ-cr-44	1982	85-110	White	82-90	18-22	17-22	1800-2400	400-700	Holetta and Indibir
DZ-cr-82	1982	96-112	White	74-98	24-28	17-22	1700-2000	300-700	Jimma and the surrounding
DZ-cr-37	1984	67-90	White	76-138	18-28	14-18	1500-2200	134-500	Low Altitude, low moisture a
DZ-cr-255	1993	63-116	White	75-137	20-30	20-25	1500-2200	500-850	Jimma and Awassa
DZ-01-974	1995	84-132	White	115-118	24-34	20-25	1600-2400	300-700	Mid to high altitude
DZ-cr-358	1995	70-109	White	125-140	21-36	15-25	1600-2400	300-700	Mid to high altitude
DZ-01-2053	1998	65-103	Brown	75-137	18-23	17-227	1900-2400	300-700	Holetta and its surrounding
DZ-01-1278	2000	85-110	White	75-137	24-35	16-22	2200-2400	300-800	Ambo area
DZ-01-1281	2002	83-100	White	73-95	22		1850	960	Sirinka and its surrounding
DZ-01-1285	2002	80-92	White	104-118	24-26		1900-2200	800-1000	Akaki and Ada , east shewa,
DZ-01-1681	2002	74-85	Dark brown	84-95	25		16ßß-1900	300-860	Dehra, low rainfall of th valley

1.2.8 The objectives of the thesis

The objectives of this Ph.D research work are:

1. Establishment of embryo/ovary rescue technique for tef and wild species and to optimize various factors in the culture media for the various stages of explant.

2. To establish an efficient in vitro protocol for immature embryo culture of tef.

3. To establish and develop techniques for the production of haploids and dihaploids from, both androgenesis and gynogenesis studies of tef.

4. To establish protocols for tef transformation studies using both biolistic and agrobacterium transformation techniques.

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Chapter 2

Literature Review

2.1 Cytogenetics of tef: Microscopic study

Tef [*Eragrostis tef* (Zucc.) Trotter] is an allotetraploid species (Berhe, 1981: Tavassoli, 1986) with the chromosome number 2n=4x=40. *Eragrostis* in general has a very small size chromosomes (Tavassoli, 1986, Gugsa, 1993) and tef has the smallest chromosome (0.8-2.9 µm) ever reported in Poaceae family members (Ayele et al., 1996: Gugsa et al., 2000). Cytological techniques for routine chromosomal analysis of both somatic and meiotic divisions in *Eragrostis* are still difficult and need further refinement.

According to Tavassoli (1986), most of the tef chromosomes are either metacentric or sub metacentric, a few are acrocentric and one or two pairs are satellited. Twenty bivalents are normally formed during diakinesis to metaphase I in pollen mother cells of tef varieties and their hybrids (Tavassoli, 1986). Subsequent mitotic stages are also normal. Unlike some of its wild relatives, chromosomal races and aneuploids are not reported in tef and this is in conformity with the absence of genome-size variation (Ayele et al., 1999) among tef varieties studied.

2.1.1 Flow cytometry analysis

According to Ayele et al., (1996) there was no polyploidy variation other than the tetraploids observed among all the tested 35 (Ebba's) tef cultivars and some released varieties including DZ-01-196 using flow cytometric analysis. Furthermore, the tetraploid genome size of tef is found to be about 730 Mbp, which is roughly 50 % larger than rice, and the equivalent of diploid tef genome size is about 75 % the size of the rice genome (Ayele et al., 1996). The genome size of the hexaploid wheat is 16000 Mbp and maize being an ancient tetraploid has a genome size of 2500 Mbp.

2.2 Tissue culture

The lack of an efficient *in vitro* culture system limits the development of a reproducible genetic transformation protocol. The main factor determining the tissue culture response in most recalcitrant species such as rye (Vasil, 1983) include genotypic differences, donor plant growth,

and developmental stage of the explants at the time of culture initiation, culture medium composition and culture conditions. In contrast most cereals highly responsive genotypes have been identified (rice; Taipei 309, IR 54; barley, Golden promise; wheat: Bob white) and reproducible tissue culture system have been developed.

Different strategies of *in vitro* culture system have been reported for tef (Bekele et al., 1995, Mekbib et al., 1997, Assefa et al., 1998, Gugsa et al., 1999). It is generally agreed that a donor plant quality plays a key role in the establishment of regenerable tissue culture and transformation success. Donor plants should be healthy and vigorously growing during seed setting. There is no universally applicable method of culture and regeneration for all species as tissues from different sources and species as well as varieties differ in their response and requirement to tissue culture. A protocol to produce embryonic callus from one culture, may be different from that of another culture, even within the same species (Vasil, 1983). Different tissues have been described for tissue culture initiation including the production of haploids. Many crops benefited from double haplodization technique however, no protocol has yet been developed for tef except one preliminary report in anther culture (Tefera et al., 1999).

Immature embryos and inflorescence were the two most frequently used explants for tissue culture in rye (Vasil, 1983), barley (Kachhwaha and Kothari, 1996), wheat (Arzani and Mirodjagh, 1999), millet (Vikrant and Rashid, 2002), Sorghum (Oldach et al., 2001) and many other crops. Since different genotypes and media compositions were applied in early reports of tef tissue culture, callus induction frequencies and regeneration response of different explants can hardly be compared. In tef like many other cereals, younger tissues of leaf, roots and inflorescence are found to be suitable using modified MS medium with various concentrations of growth hormones (Assefa et al., 2000). The developmental stage of explants has been found to be crucial factor in the establishment of totipotent cultures (Vasil and Vasil, 1986). Donor plants with immature inflorescence can be harvested at a relatively early developmental stage and might therefore, escape unfavourable environmental conditions that apply to those that produce immature explants.

An in-depth study of the effect of genotypes explants and factors that affect tissue culture responses would enable the development of genotype specific culture protocol. One further

important factor is the culture length, which can affect the reproducibility of a culture protocol (Vasil and Vasil, 1986). Efficient and short culture period are very important for large scale plant multiplication and transformation. High auxin levels decrease the regeneration potential and should therefore be reduced and kept as low as possible in the regeneration medium. Several other cultural factors like temperature during *in vitro* culture or callus culture under dark or light conditions (Eapen and Rao, 1983) also influence the tissue culture performance and should be taken into account when establishing an *in vitro* culture system.

2.2.1 Cell and tissue culture and its application in cereal improvement including tef.

Plant regeneration in tissue culture is a crucial aspect in plant biotechnology as it facilitates the production of genetically engineered plants, the release of disease–free plants from meristem cultures and the rapid multiplication of difficult species to propagate them. Plant regeneration through tissue culture can be accomplished by enhanced auxiliary branching from shoots or lateral buds (micro propagation), somatic embryogenesis, embryo culture or organogenesis (Vasil, 1985; 1986; Thorpe and Statsolla, 2001).

Plant breeding has been useful as a means of increasing yield, disease resistance, nutritional quality, and other agronomic traits in cereal crops including barley, maize wheat, rice and sorghum. Now a days *in vitro* techniques have been routinely used for crop improvement. This includes micropropagation, regeneration from protoplasts (Vasil, 1987), somatic embryogenesis (Thorpe and Statsolla, 2001), haplodization (Touraev et al., 1997), in vitro fertilization, embryo rescue (Smith, 1971), and so on. Establishment of plant cell and tissue culture techniques for tef was established only by using seed culture (Assefa et al,, 1998), young leaf base (Mekbib et al,, 1997) and root culture (Bekele et al., 1995). However, no improved variety was obtained so far from this technique. Therefore, further work is needed for a successful tef improvement through tissue culture system and genetic transformation.

Haploid or dihaploid plants production through anther or microspore culture currently is used by many commercial companies for breeding purposes as an alternative to the numerous cycles of inbreeding as well as backcrossing in conventional methods. The efficiency of anther and microspore culture in producing a new variety is directly related to the frequency of pollen plant formation (Datta, 2001). The larger the number of regenerants the higher is the chance to obtain the desired genotypes. There are also additional advantage of stability and uniformity of the new

varieties. However, various factors affect the application of this technique (Chen, 1986; Gu et al., 1978; Datta, 2001). Since tef is a self pollinated annual plant, the conventional breeding methods needs a minimum of 12 years from the stage of crossing or selection to a serious of field evaluation and variety release. Establishing an efficient method of *in vitro* plant regeneration through haploid plant production would exhibit a fast and impressive result in tef improvement research. However, this technique is not developed for tef.

2.2.2 Somaclonal and gametoclonal variations

Somaclonal and gametoclonal variations can be considered as additional option to generate genetic variability in co-adapted, agronomically useful cultivars without the need to resort to hybridization and so far have provided breeders with new sources of variability to incorporate into conventional breeding programmes (Evans and Sharp, 1985). Plants with useful agronomically traits such as increased tolerance to physiological stress, pests and diseases and herbicide resistance have been recovered from such materials (Bajaj, 1990). According to Picard and Buyser (1977) many wheat double haploid (DH) gametoclonal variant lines, mutate to resistance to powdery mildew and septoria, high grain yield, panicle weight and length and tiller number.

Production of somaclonal variants will allow selection of distinct phenotypes (cell lines) from a large population of cells coupled with *in vitro* mutation techniques. Genetic improvements for characters which can be identified at the cellular level such as enhanced resistance to an adverse factor in the cell's microenvironment might best be tackled by mutation and direct selection in cell cultures (Dix, 1986). Such characters would include resistance to diseases pathotoxins and environmental or chemical stress. Somaclonal variation for other characters for which there is no adequately defined *in vitro* response, such as seed protein quality, photosynthetic efficiency, or yield can be utilized provided effective whole plant screening and regeneration procedures are available (Evans and Sharp, 1985).

2.2.3 Spikelet culture

A system was developed to investigate the *in vitro* manipulation of the spikelets of tef for self pollination, formation of kernels and germination *in vitro* (Tefera and Chapman, (1992). According to their results, tef spikelets developed successfully in wheat spikelet medium.

Furthermore, spikelets of the F_1 varieties (Fesho X Kaye Murri) showed good early development. This might be developed as an alternative to the current surgical hybridization (which is slow and time consuming) by extending the study on *in vitro* pollination and fertilization of tef flowers. This result should further be strengthened by extending the investigation to other tef varieties and cultivars.

2.2.4 *In vitro* **regeneration (somatic embryogenesis and organogenesis):** Although, some studies have been done in tef plant regeneration through *in vitro* culture, only Assefa et al., (1998) and Gugsa et. al., (1999) reported high regeneration (87-93 %) from seed compared to 2-3 % from young leaf (Mekbib et al., 1997) and root basis (Bekele et al., 1995) mainly due to differences in the explant types, media compositions and genotypes used. The efficiency of plant regeneration should be further improved. Because, transformation system requires maximum regeneration from competent tissue, immature embryos are reported to be the very efficient explants for high regeneration capacity for use in transformation studies (Becker et al., 1994). A comparative account of somatic embryogenesis from immature embryos is lacking in *E.tef.* Micropropagation or regeneration of tef plantlets through *in vitro* multiplication is also important to clone the F_1 hybrids which would be rarely obtained particularly from interspesific crosses of tef.

2.2.5 Protoplast fusion: It has long been proposed as an innovative and far-reaching method to produce hybrid plants that can't be obtained by sexual means. The best use of protoplast fusion will be in the production of cybrids which contain the nuclear and cytoplasmic genome of one and only the cytoplasmic genome of the second parent (Vasil, 1983). These can be of particular advantage to transfer cytoplasmic male sterility, an important trait in tef breeding.

Somatic hybridization through protoplast fusion of *E.tef* and *Sorghum bicolor* was attempted using electric field mediated fusion (Bekele, 1995). Survival and division of a small fraction of cultured protoplasts and protoplast fusion products were observed using heat shock treatments that stimulated cell wall formation and cell division in both sorghum and tef. However, regeneration from callus of the fused products of tef and sorghum has not yet been reported.

2.3 Embryogenesis

An embryo can be defined as the earliest recognizable multicellular stage of an individual that occurs before it has developed the structures or organs characteristic of a given species (Gray, 1996). Therefore, embryogenesis is the development of an embryo till its maturity. In natural condition, embryogenesis affects the transition from the fertilized egg to the new multicultural generation, the seedling, which displays the basic body plan and organization of the plant. There are three types of embryogenesis, zygotic, somatic and gametic where, the last two embryo types resemble the zygotic embryos but derived from the somatic and gametic cells respectively.

2.3.1 Zygotic embryogenesis: Zygotogenesis is the starting point of embryogenesis the formation of zygotic embryos after the fusion of male and female gametes *in vivo* or *in vitro* (Czapik and Izmailow, 2001). Zygotes, the first cells of the sporophytic generation are products of fertilization and they show dynamic changes in their morphology and physiology soon after fertilization and they start their characteristic type of development (embryogenesis) which if continues undisturbed by external and internal factors, secures normal germination and post-germination development (Czapik and Izmailow, 2001). The differentiation of the zygote into an embryo capable of germination is a key process in the life cycle of plants (Fisher-Jglesia and Neuhaus, 2001). There are two types of zygotic embryogenesis systems, monocotyledonous and dicotyledonous and they have distinct structural and physiological process differences. Several studies have addressed the *in vitro* zygotic embryogenesis, where embryo rescue is the major topic.

2.3.1.1 Embryo rescue: One of the uses of tissue culture is to rescue immature embryos from the natural effect of incompatible wide or inter-specific crosses to produce a new hybrid that may not be possible in natural conditions. This technique is a well established branch of plant tissue culture that allows the development and germination of zygotic embryos from early stage to mature stage of development (Raghavan, 1965, 1977; Norstog, 1979; Hu and Wang, 1986). Wide hybridization between two distinct species normally fails because of fertilization barriers, e.g. embryo and endosperm lethality or disruption of embryo-endosperm relations (Simmond, 1979, Brar and Khush, 1986). If an embryo-endosperm relation is the disrupting agent, then embryo culture can be very valuable technique and indeed such embryos of several plant species have

been rescued. A hybrid between *E.tef* (2n=4x=40) and *E.cilinensise* (2n=6x=60) can be an example where the hybrid seed fails to germinate (Tavassoli, 1986) but could have been rescued if an appropriate embryo rescue technique were established.

The improvement of tef is technically hindered due to the tedious crossing technique as was presented in Chapter 1 and this is accompanied by the unique pollination behaviour of tef and its weak floral organs that are easily damaged mechanically after *in vivo* pollination. Lodging and drought are the most prominent factors for low yield in tef (Ketema, 1983). This could be improved by recombining genes from the wild species. However, this was hindered due to the failure of seed setting in almost all interspecific hybridization. Recently Gugsa et al., (1999) reported 75 % seed setting between the cross of tef and *E.pilosa* cv. 30.5 which has high sterility of the F_1 hybrids especially when the mother plant used was the wild species. The failure of this and other interspecific crosses could be due to post fertilization barriers like embryo abortion due to endosperm- embryo incompatibility or incomplete development of endosperm.

Embryo-rescue technique development in tef was attempted by Cheverton (1985) using mature embryos. Here seeds were allowed to imbibe water overnight and then surface sterilized with 0.5 % sodium hypochlorite. Embryos were excised with a fine forceps under a binocular microscope; the seed being restrained by the surface tension of a drop of water in a hydrophobic petri dish, such embryos grew rapidly both on half strength and full strength MS (Murashige and Skoog, 1962) medium (Cheverton, 1985). However, it is difficult to consider or use the technique for embryo rescue method since the stage of embryo development plays a vital role i.e. embryos should be rescued before abortion. Besides, it is known that in many interspecific crosses, seeds can not be developed further to reach mature embryo stage; the zygotic embryos may abort at earlier stage. Thus, in tef a protocol which permits the development and growth of an immaturely excised zygotic embryos or ovaries, few days after pollination is required.

Embryo rescue using immature explants such as ovules, embryos and sliced ovaries have been reported in many angiosperms and gymnosperms**.** In oil crops such as; *brassica* (Ross, 1980), flax (Pretova, 1983)**,** in horticultural crops; tomato (Smith, 1944), lilium (Hai-Shan, 2002; Kanoh et al., 1988), interspecific hybrids of allium (Dolezel et al., 1980); in legumes**;** faba bean (Smith,

1971, Theano et al., 1993), pasture legume (William, 1980), in cereals; barley (Cameron-Mills et al., 1977; Umbeck and Norstog, 1979; Bürün, 2002), maize (Burghardtova, 1980; King and Shimamoto, 1984), Wheat (Kranz and Kumlehn, 1999; Kumlehn et al., 1997), rice (Shame and Bhaduri, 1982) and sorghum (Gamborge et al., 1977). Kumlehn et al., (1998) reported an efficient and reproducible embryo *in vitro* development obtained from fertilized wheat egg cells isolated 3-6 h after hand pollination. They also showed a successful development of wheat embryo to a plant via zygotic ovule culture (Kumlehn et at., 1997).

Although, there are many established embryo rescue protocols for various crops, it is not possible to use all protocols to other species since many factors including genotype, culture medium, culture condition; immature explant type and age play a role in the success of the rescue. The excision of the immature embryos and ovaries of tef may be difficult, since matured tef seeds themselves are 1.0-1.6 mm in size (Ebba, 1975). Besides, the most important aspect in culturing immature embryos is to define a culture medium that can sustain their growth and development. Under sub optimum medium, the immature embryos may fail to survive, turn into undifferentiated callus or germinates prematurely (precocious germination) and such germination results in weak seedlings (Hu and Zanettini, 1995) or some time the hybrid may die. Therefore, *in vitro* regeneration system using immature explants or young seedling of hybrids is required. MS (Murashige and Skoog, 1962), Gamborge B5 (Gamborge et al., 1968) and White (White, 1963) media with certain degrees of modification are the most widely used basal media in embryo culture (Hu and Wang, 1986).

2.3.2 Somatic embryogenesis

Somatic embryogenesis is defined as a process in which a bipolar structure resembling a zygotic embryo develops from a non zygotic cell without vascular connection with the original tissue (Von et al, 2002). It is a process by which somatic cells differentiate into somatic embryos. Somatic embryos are bipolar and bear typical embryogenic organs, the radicle, hypocotyl and cotyledon. Therefore, during somatic embryo (SE) development in dicots and monocots, stages equivalent to the globular, heart, torpedo stages of zygotic embryogenesis can be found similarly with globular, coleoptilar and scutellar stages of monocots (Thorpe and Stasolla, 2001). Somatic embryos can probably be achieved for all plant species provided that the appropriate conditions

like explant, culture media and environmental conditions are employed. According to Von et al., (2002) somatic embryogenesis is a multi-step regeneration process starting with the formation of proembryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration.

Somatic embryos are used as a model system in embryological studies. However, the greatest importance of somatic embryos is its practical application in large scale vegetative propagation. In such cases somatic embryogenesis is favoured over other methods of vegetative propagation because of the possibility to scale up the propagation by using bioreactors (Von et al., 2002). Embryogenesis is a complex process in which the quality of the product i.e. survival and growth of regenerants depend on the conditions provided at earlier stage, when mature somatic embryos are formed and germinated. (Von et al, 2002). Various plant parts have been used to produce somatic embryos. For many species, the explant choice is the zygotic embryo, which consists of cells already possessing embryogenic competence, termed pre-embryogenic determined cells (Evans et al., 1981).

During the last decade an impressive progress has been made on plant regeneration from immature embryos of major grass species and cereal crops (Vasil and Vasil, 1986; Vishnoi and Kothari, 1995; Kachhwaha and Kothari, 1996; Arzani and Mirodjagh, 1999; Folling and Olesen, 2001; Oldach et al., 2001; Kennedy et al., 2001). Immature embryos were reported to be better than mature embryos due to their suitability for high somatic embryo formation (Vikrant and Rashid, 2002). Establishing a reproducible *in vitro* culture protocol is essential for each genotype and explant type since the responses of tissues are dependent on the various culture conditions among which medium composition exerts the largest effect. Earlier studies indicated that for many species an exogenous auxin was an absolute requirement to induce embryogenic cells (ECs) all referred to as embryogenic masses or clumps, proembryos, proembryonic tissue, etc. Embryogenic culture is also an attractive target for genetic transformation. In other cereals immature embryos of scutella tissues of wheat (Becker et al., 1994), maize, (Brettschneider et al., 1997), barley (Chang et al., 2003), sorghum, (Brettell et al., 1980; Ma et al., 1987; Oldach et al., 2001), rice (Cho et al., 2004) were reported to be the very efficient explants for high regeneration capacity. In addition, in most cases the somatic embryos or the embryogenic cultures can be cryopreserved, which makes it possible to establish gene banks (Von, et al., 2002).

2.3.3 Gametic embryogenesis: Gametic cells which already exist as egg cell or microspore in a single cell system in nature can develop to embryos. Like sporophytic cells, gametophytic cells can also be regenerated into functional plants (Datta, 2001). These embryos are derived from either the male (microspore and/or anther) or female (ovary, ovule, egg cell, synergies, and antipodal) gametes *in vivo* or *in vitro.*

The main advantage of using haploidization is the rapid and complete homozygosity of the offspring for phenotype selection. By doubling the chromosome number using chemicals or as it occurs spontaneously, di-haploid (double haploid, DHs) can be achieved so it greatly reduces the time required for development of improved varieties.

There are many techniques to produce haploids in plants among which (i) genomic elimination via wide hybridization, ii) androgenesis and iii) gynogenesis are commonly used.

2.3.3.1 Wide hybridization: The first report of *in vivo* haploid production was obtained by (Kasha and Kao, 1970) via wide hybridization of *Hordeum vulagare* with *Hordeum bulbosum* which is termed as bulbosum technique (chromosome elimination). In these crosses the chromosomes of the *Hordeum bulbosum* were eliminated during mitosis division and only

Hordeum vulagare's chromosomes remained to produce haploid (Simmonds, 1979). Hybrids between *H.vulgare* and *T.astivem* (Islam et al., 1975; Snape et al., 1980), and wheat with maize pollination (Suenaga and Nakajima, 1993) were produced with no serious barrier. However crosses between corn and sorghum and sorghum and pearl millet (Reger et al, 1982) showed less success. The routine techniques in doubled haploids (DHs) production of many cereals crops are from *in vitro* androgenesis.

2.3.3.2 Androgenesis: Since the successful report of androgenic haploids of Datura (Guha and Mahesnwari, 1964) and rice (Niizeki and Oono, 1968) great effort have been done in the *in vitro* production of haploid plants. For instance, in wheat, anther culture (Barnabas et al., 2001) or isolated microspore culture (Zheng et al., 2001; Hönicka, 2001) had resulted in double haploids.
Androgenesis is a system of achieving the regeneration of complete plants through male gametes, anther or microspore culture *in vitro*. In many plant species pollen or microspores develop into haploid plants via embryogenesis *in vitro*. The process mostly requires the switch from the normal development of pollen grain to sporophytic mode of development through several types of pre-treatment so that instead of male gametes, plants are produced in culture (Maheshwari et al., 1982; Jähne and Lörz, 1995; Kumlehn and Lörz, 1999).
Pollen embryogenesis offers an opportunity to investigate the cellular physiological, biochemical and molecular process of totipotency using isolated single cells that can be readily staged for analysis (Reynolds, 1990; Kumlehn and Lörz, 1999). Moreover, pollen represents an interesting experimental system for developmental biology because pollen is the only tissue in higher plants which consists of isolated cells without cell wall connections for the most part of its development (Heberle-Bors, 1998). This remarkable potential for cellular totipotency from male gametophyte is called androgenesis or pollen embryogenesis (Reynolds, 1990).
Hu and Guo, (1999) reported that *in vitro* androgenesis aided the development of several improved varieties. For instance more than 38 new rice; 22 maize and 10 wheat varieties were released from androgenesis.

Anther or microspore culture

Anthers contain pollen and anther culture involves the culture of these structures *in vitro* and microspore culture is the culturing of mechanically isolated microspores from the immature

anther. The immature pollens or microspores contained in the anther either gives rise directly to embryos called androgenesis or to callus tissue which in turn is induced to regenerate plants under the influence of growth hormones that are added to the culture media (Jähne and Lörz, 1995).

According to Toureav et al., (1997) and Heberle-Bors (1998) the development of pollen follows a tightly controlled sequence of events that can be divided into two major processes, microsporogenesis and microgametogenesis. Microsporogenesis begins with meiosis and ends with the formation of polarized haploid microspores. During microgametogenesis the unicellular microspore divided asymmetrically, resulting in a young pollen grain containing a vegetative cell and generative cell that differentiates into mature bicellular or tricellular pollen grains (Bedinger, 1992; Toureav et al., 1997; Touraev et al., 1995). In microgametogenesis immature pollen or microspores enter an alternate developmental pathway in which microspores initiate indeterminate growth giving rise to mature plants with the haploid (gametic) number of chromosomes (See Reynolds, 1990; Maheshwari et al., 1982; Kumlehn and Lörz, 1999). This microspore embryogenesis, irrespective of whether the embryos originate from unicellular microspores or immature bicellular pollen grains gives haploid plants that could rise from induction of pollen plant through anther culture or isolated microspores. This has been successful for many plant species including cereals. Jähne and Lörz (1995) had reviewed the cereal microspore culture in detail.

In cereals the most common form of microspore culture was the isolation of intact anthers because it is a relatively simple and fast method and requires only minimal facilities (Jähne and Lörz, 1995). However, the use of isolated microspore would have several advantages over anther culture. This include manipulation of a single cell populations, removal of undesirable interactions between the anther wall and the microspore obtaining ample material for replicated experimentation and the possibility of obtaining haploids and a better access to biotechnological applications such as *in vitro* selection (Swanson et al., 1989) and transformation (Kumlehn, 2005 personal communication).

2.3.3.3 Gynogenesis: The second method of gametic embryogenesis is by using the female gametes and is called gynogenesis. One of the interesting peculiarities in unpollinated ovary

cultures is the possible induction of some elements of apomixes without pollination. In some apomictic plants, the egg cell apparently divides in total absence of a stimulus from male gametes (Bantin et al., 2001). In several other plants it is possible to stimulate the unfertilized egg cell and or some other cell of the embryo sac (apogamy), by external treatments, so that division and formation of embryo results (Lacadena, 1974; San and Gelebart, 1986).

Normally the egg cell requires the fertilization stimulus to enter embryogenic development. However, it has the necessary genetic information to initiate embryogenesis independent of fertilization (parthenogenesis). Cells of embryo sac and the somatic tissue surrounding it can proceed from the final differentiation state and form apomictic embryos in natural (Bantin et al., 2001) and *in vitro* conditions (Kazakhstan, 1996). In most cases of gynogenesis, external stimuli applied to the gynogenic cells are required. Some of these stimuli include low temperature shock, application of growth regulator or chemicals, delayed or early pollination, and pollination with pollen in which the normal development of male gamete has been disturbed by irradiation or chemical treatment (Sestili and Ficcadenti, 1996).

Despite the limited explants of female organs compared to microspores, gynogenesis is used as an alternative technique to produce haploid and di-haploids. San (1976) was the first to report gynogenic haploids in unfertilized ovary culture of barley. Since then *in vitro* gynogenesis has been reported in at least 23 species mainly of horticultural crops (Bhojwani and Thomas, 2001). Plant regeneration from gynogenic explants has been reported in many crops including cereals. For instance, *Hordeum vulgare* (San, 1976, Castillo and Cistue, 1993), *Triticum astivum* (Zhu. et al., 1981), Durum wheat (Sibi et al., 2001), *Oryza sativa* (Kuo, 1982; Zhou and Yang, 1980) and *Zea mays* (Truong-Andrei and Demarly, 1984).

Thus gynogenesis is an attractive approach to produce haploids plants where androgenesis is either not applicable or unsuccessful. Therefore, the development of an efficient *in vitro* culture and regeneration technique from gynogenic cells of tef was attempted in the present study.

2.3.4 Biotechnological approach for tef improvement: Biotechnology represents one of the latest frontiers of scientific progress of the last century. These new biotechnology could play a decisive role in agriculture because of its ability to directly modify plants, animals and agricultural processes to new needs. This technology should be seen not only as a means of solving problems when traditional techniques fail, but also as a way of generating a better

understanding of crop plants through the cooperation of scientists from different disciplines. One of the applications of biotechnology that is currently being used in crop production is genetic transformation. Transformation of plants, in general, is achieved by introducing a gene encoding a given trait, the expression of which alters the plant phenotype, through the use of several DNA transfer techniques. In the review of Potrykus (1990), fifteen different transformation methods were listed. Among these, the use of *Agrobacterium tumefaciens* has been developed to be a convenient and efficient technique. The severe limitation which was visible with the *A. tumefaciens* system is its low efficiency to transfer genes to graminaceous monocots due to host range preference of the agrobacterium. However, this may not be the main problem at least in some crops since several strains are now available to initiate virulence with monocots. Thus, the most limiting factor may be efficient regeneration from monocots.

The development of a genetic transformation system for tef and oats was recommended (CTA/FAO, 1989). However, little has been done in tef transformation studies (Mengiste, 1991) and Mekbib et. al., 2001). Mengiste (1991) reported the transient expression of the GUS gene in embryogenic callus culture of tef together with the antibiotic sensitivity of cultured tissues of tef to Kanamycin and Genticine (antibiotics). Later, Mekbib et al., (2001) reported *Agrobacterium tumefaciens* attachment as a factor in transformation using various explants and crops such as tef zygotic embryos, seeds, leaf base and embryonic callus, leaf discs of yam and tobacco species. According to these authors, although attachment was observed to all explants used, attachment was mainly observed around the wounded areas. The bacteria strain used equally bound with tef and yam in the same way as they did to tobacco which was a positive control. Thus tef and yam like that of tobacco fit all the criteria for agorbacterium attachments.

Although it is clear now that *Agrobacterium* is capable of transferring DNA to monocotyledons plants including agronomically important cereals, if tissue containing `competent´ cells are infected (Hiei et al., 1994; Chang et al., 1997; Tingay et al., 1997), but efficient transformation appears to be strongly genotype-dependent.

In comparison to biolistic method of transformation, advantages of the *agrobacterium*-mediated transformation include the transfer of pieces of DNA with defined ends with minimal rearrangements, the transfer of relatively large segments of DNA, the integration of small number of copies of genes into plant chromosomes and high quality and fertility of transgenic plants (Gallie, 1998). In addition, *Agrobacterium* mediated transformation has

been considered a very simple, cost effective and highly efficient alternative to direct gene delivery methods.

The other widely used technique for potential transformation is the use of high velocity microprojectile to deliver DNA into the intact plant cell in a non-biological system. The microscopic metal particles (mostly gold) coated with DNA are explosively accelerated into the plant cell. Thus, the meroblastic particle penetrate cell walls and may even traverse entire cells and the DNA coating may be uploaded as the particle passes through the nuclei or organelles (Singh and Shaw, 1992). This simple process is independent of biological vectors and hence, is not impeded by host range complications and has been very efficient in producing transient expression signals in many crop species. The reporter gene constructs can be delivered to various plant tissues; leaves, seedlings, shoot apices, floral tissues, pollen, microspores, and recently effectively and widely used embryonic calli derived from immature embryos or freshly isolated immature embryos. Stable transformation of wheat embryos by particle bombardment was reported by many authors including (Becker et al., 1994; Becker et al., 1997).

In any transformation system, only a small proportion of plant cells are transformed by the currently available delivery systems and, therefore, it is crucial to have a selectable marker to recognize the transformed cells and suppress the growth of wild type cells (Mengiste, 1991). Thus screening the treated tissues in a medium containing a selection agent is needed. Because sensitivity affects the recovery of transformed plants and varies widely among tissues and species used antibiotic sensitivity should be determined in the initial stages of development of a plant transformation system.

The first results in tef transformation (Mengiste, 1991) indicated that foreign DNA can be delivered to the various tissues of tef (embryogenic callus, zygotic embryos, and suspension culture cells) by the particle bombardment device. Also it has been shown that cauliflower mosaic virus (CaMv) 35S promoter is a useful promoter for gene expression studies in tef (Mengiste, 1991) and should be useful in obtaining stable transformation. In the present study the antibiotic and herbicide sensitivity levels, the appropriate stage of explant, the period of agro-stop and resting periods for agrobacterium and biolistic gene transfer methods respectively were tested.

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Chapter 3

Embryo and ovary rescue in tef [*Eragrostis tef* (Zucc.) Trotter]

Zygotic Embryogenesis

3.1 Materials and Methods

3.1.1 Plant materials: Six genotypes were used for this study. Three tef varieties, which include the widely grown improved (DZ-01-196 and DZ-CR-37) and the land race, (Fesho) and three wild *Eragrostis* species. (*E. pilosa*, *E. mexicana* and *E.papposa).* Seeds were germinated in medium sized pots containing peat soil mix in a greenhouse of Hamburg University, at the Institüt of Biozentrum, Klein Flottbek und Botanischer Garten, (AMPII), Germany, at 26/18°C day/night, 16 hr photoperiod and 70 % relative humidity. Fertilizer, Plantosan (Aglucon, Dusseldorf, Germany) 12 g/pot, N.P.K 15:8:15 was applied once, after three weeks of potting. At early to mid anthesis stage, the *in vivo* growth and developmental stages of the self fertilized ovaries and embryos were estimated by repeated observation especially from a genotype (DZ-01-196), which has a red colour anther used as marker.

3.1.2 Surface sterilization: Panicles containing immature caryopsis, 1-9 days after pollination (dap) were cut and immersed in flask containing water prior to further processing in the laboratory. Surface sterilization of the cut panicle was performed by inserting in a 50 ml plastic tube containing 1 % sodium hypochlorite, vortexed for 15 minutes and rinsed 3-4 times with sterile de-ionized water.

3.1.3 Isolation of pollinated ovary: Self fertilized pistils/ovaries at the age of 1-2, 3-5, 6-7 and 8-9 dap) respectively were isolated from the florets (Figs. 3a, 3b and 3c). The florets were opened using fine forceps and scalpel under a binocular microscope asceptically and the pollinated ovaries were collected on a moist glass dish. The collected explants were grouped according to their size for in vitro culture.

3.1.4 Isolation of immature embryo: The immature zygotic embryos were excised using the following procedure**:** Immature caryopsis (5-12 dap) were collected and categorized as small (S),

intermediate (I) and large (L) sizes. These were laid on a sterilized glass dish under a binocular microscope (position of the embryo faced up, Fig. 3b arrow) and by holding one side of the immature caryopsis with fine forceps, the tip of the micropylar end of the pollinated ovaries was cut with fine blade. Then slightly the embryos were pushed towards the cut and were picked by the tip of the blade to immediately transfer the embryos to culture medium. Quick capture of the embryos using the flat side of the blade and plating on the medium is essential to protect them from fast desiccation.

3.1.5 Media: Only K99 medium (Table 4) devoid of hormones (K99ER) was used solely to rescue both immature ovaries and embryos after the preliminary result of using modified MS (Murashige and Skooge, 1962) and N6 (Chu, 1978) media. The effect of the various concentrations of glutamine (0, 1, 3, 6 and 10 mM) and maltose (150, 250 and 350 mM) levels were compared. Besides, addition of 1 mg/l of GA_3, in the germination medium was compared with the control to see the effect of this hormone on the germination capacity of the cultured explants.

3.1.6 Culture condition: Explants with various ages were cultured separately according to their sizes .The zygotic ovaries were cultured at upright position by immersing the base of the ovaries in the solid medium. The embryos were cultured as the scutelum touching the surface of the medium. Ten to fifteen explants were cultured in each disposable plastic petri dish (35 x 10 mm), with a minimum of 60-75 embryos per treatment for each experiment. The cultures were incubated in dark at 25°C for 5-10 and 14-30 days for ovaries and embryos, respectively unless other wise stated. Later the embryos were transferred to the germination media and cultured in the light (16/8 h) until good germination (roots and shoots) was established. During the transfer of the immature caryopsis to the germination medium, the position of the embryos in the developing ovaries was directed to the light. Germinated plants were transplanted to pots filled with soil and grown in the green house.

3.1.7 *In vitro* fertilization: Intra and interspecific crosses were attempted in aseptic condition using two tef varieties, DZ-01-196 and DZ-CR-37 and two wild species, *E.mexicana* and *E. papposa* (Table 16). Florets were emasculated and the freshly isolated, matured anthers were

collected on moist tissue paper in glass petri dishes and stored under humid condition. Matured anthers and receptive pistils (with widely opened stigmas) were excised from the mother plant and plated on embryo rescue media with the stigma positioned upright.

The pollen grains were dispersed from the collected anthers and summed on the tip of a needle. *In vitro* pollination was done by carefully applying the pollen grains on the surfaces of the cultured stigmas. Petri dishes were sealed, labelled and incubated both at the dark and light conditions.

3.1.8 *In vivo* crossing:

For wide crossing, more than hundred florets of variety DZ-01-196 were chosen and surgically opened to isolate the matured (ready to pollinate) pistils, which were one day before self pollination. These were pollinated with freshly collected matured pollen of maize at *in vivo* condition. After opening the tef florets, maize and wheat pollen was immediately dispersed on the surface of the stigmas using the tip of the fine blades. Six to 12 hours after pollination the panicles were harvested, surface sterilized and aseptically the pollinated pistils were isolated and cultured using the embryo rescue medium (K99ER). The materials used for the crossing were described in Table 16.

A number of experiments were carried out to sustain the growth of the excised explants and increase of the rescue efficiency. These include:

Experiment 1. The effect of various levels of glutamine (0, 1, 3, 6 and 10 mM)

Experiment 2. The effect of the age of ovaries: 1-2, 3-5, 6-7 & 8-9 dap and sizes of embryos; S, small (30-50 µm), 5-7 dap; I, intermediate (51-150 µm), 7-9 dap and L, large (151- 350 µm), 8-12 dap

Experiment 3. The effect of explants types (ovaries and embryos)

Experiment 4. The effect of maltose concentrations (150, 250 and 350 mM)

Experiment 5. The effect of culture duration in the dark for both explants (0, 3, 5, 7, 10, 12, 15, 20, 25 and 30 days)

Experiment 6. The effect of GA_3 on germination medium (GM)

Experiment 7. The responses of genotypes

Table 4. Nutrient composition and concentration of K99 basal medium and K4NB (regeneration medium).

Components	**Concentration, mg/l and in (M unit)**	
	K99 basal medium	**K4NB regeneration medium**
Inorganic salts		**K4N Macro**
$(NH4)_2SO_4$	80 (1 mM)	320 (4 mM)
KNO_3	2,022 (20 mM)	3640 (36 mM)
KH_2PO_4	340 (2.5 mM)	340 (2.5 mM)
$CaCl_2.2H_2O$	441 (3 mM)	441 (3 mM)
$MgSO_4.7\ H_2O$	246 (1 mM)	246 (1 mM)
NaFeEDTA	27.5 (75 µM)	27.5 (75 µM)
$MnSO_4.4\ H_2O$	11.2 (50 µM)	11.2 (50 µM)
H_2BO3	3.1(50 µM)	3.1 (50 µM)
$ZnSO_4.7\ H_2O$	7.2 (25 µM)	7.2 (2 5µM)
$Na_2MoO_4.2\ H_2O$	0.125 (5 µM)	0.12 (0.5 µM)
$CuSo_4.5\ H_2O$	0.025 (0.2 µM)	1.25 (5 µM)
$CoCl_2.6\ H_2O$	0.025 (0.2 µM)	0.024 (0.1 µM)
KI	0.17 (1 µM)	0.17 (1 µM)
organics	(Sifgma K-3120)	
Retinol	1.01 (0.04 µM)	
Thiamine HCl	1.0 µM	10..0 µM
Riboflavin	0.2 (0.5 µM)	
Ca-panththenate	1.0 (4.2 µM)	
Folic acid	0.4 (0.9 µM)	
Pyridoxine HCl	1.0 (4.9 µM)	
Cobalamine	0.02 (µM)	
Ascorbic acid	2.0 (11.4 µM)	
Calciferol	0.01 (0.03 µM)	
Biotin	0.01 (0.04µM)	
Cholin chloride	1.0 (7.1 µM)	
p-aminobenzoic acid	0.02 (0.1 µM))	
myo-inositol	100 (0.6 µM)	
pyradoxine HCl	1.0 (4.9 µM)	1.0 µM
Nicotinic acid	1.0 (8.1 µM)	
Organic acids		
Malic acid	40 (0.3 µM)	
Citric acid	40 (0.1 µM)	
Fumaric acid	40 (0.3 µM)	
Na-pyravote	20 (0.2 µM)	
Glutamine	1.023 (7mM)	0.25 M
Casin hydrolysate	250 µM	
$CuSO_4$		10 mM
MES	213.2 (1M)	
MaltoseH_2O	90,000 (250 mM)	36,000 (100 mM))
2,4-D	2.17 (10 µM)	BAP (1 mM)
Phytagel	4000	4000
Zeatin	-	5.5 (25 µM)
pH	5.8	5.8

Reference: Kumlehn unpublished

3.1.9 Experimental design: Three experiments were conducted for each variable using three replications and analysed either in a completely randomized design (CRD) and statistically analysed by constructing ANOVA Table using Duncan's method or the mean ±SD. was calculated to analyse the significant differences at 0.05 probability levels. Besides, percent of normal germination were calculated out of the total number of explants cultured.

3.2 Results

3.2.1 Isolation of immature zygotic ovaries: Immature ovary/embryo isolation technique of tef and its wild relatives was established successfully for a routine application and use. The developmental stage of the fertilized ovaries was estimated (1-12 days) after self pollination. The easy and distinctive features to identify the age of ovaries were the sizes and the colour of the top and basal edges of the ovaries. For instance, the 1-2 dap ovaries were only a bit larger than the matured unpollinated ovaries and has invisible or very light green tips. The 3-5 dap, has light green tip and enlarged longitudinally and ovaries are transparent (watery). The 5-7 dap, have deep green tips and are developed to milky white. The estimated growth stages of ovaries are as described in Figs.3a-d.

3.2.2 Isolation of immature zygotic embryos: Attempt to excise immature zygotic embryos (50-100 µm) out of the immature caryopsis was difficult at the beginning. However, after repeated trial, it was possible to excise more than 100 embryos per day. The extremely small embryos (20-30 µm, Fig 4a) mostly run-out with the endosperm fluid or ovule sap and are hardly seen unless cautiously handled. Therefore, quick capture of these embryos using the flat side of the blade and plating on the medium is essential to protect them from the fast desiccation and the loss of the embryos. However, it was very tiresome and tedious to culture many of these sizes.

The small, 5-7 dap (Fig 4b), intermediate, 7-9 dap (Fig. 4c left) and the large (8-12 dap) (Fig. 4c right) stages of the immature zygotic embryos are easily seen and can be separated from the ovaries. Especially the intermediate stage embryos are easy to excise. On the other hand, the matured embryos (Fig. 4d) from the physiologically matured caryopsis are very large in size (app. 0.8 mm) and during excision they stick to the endosperm and were hard to isolate.

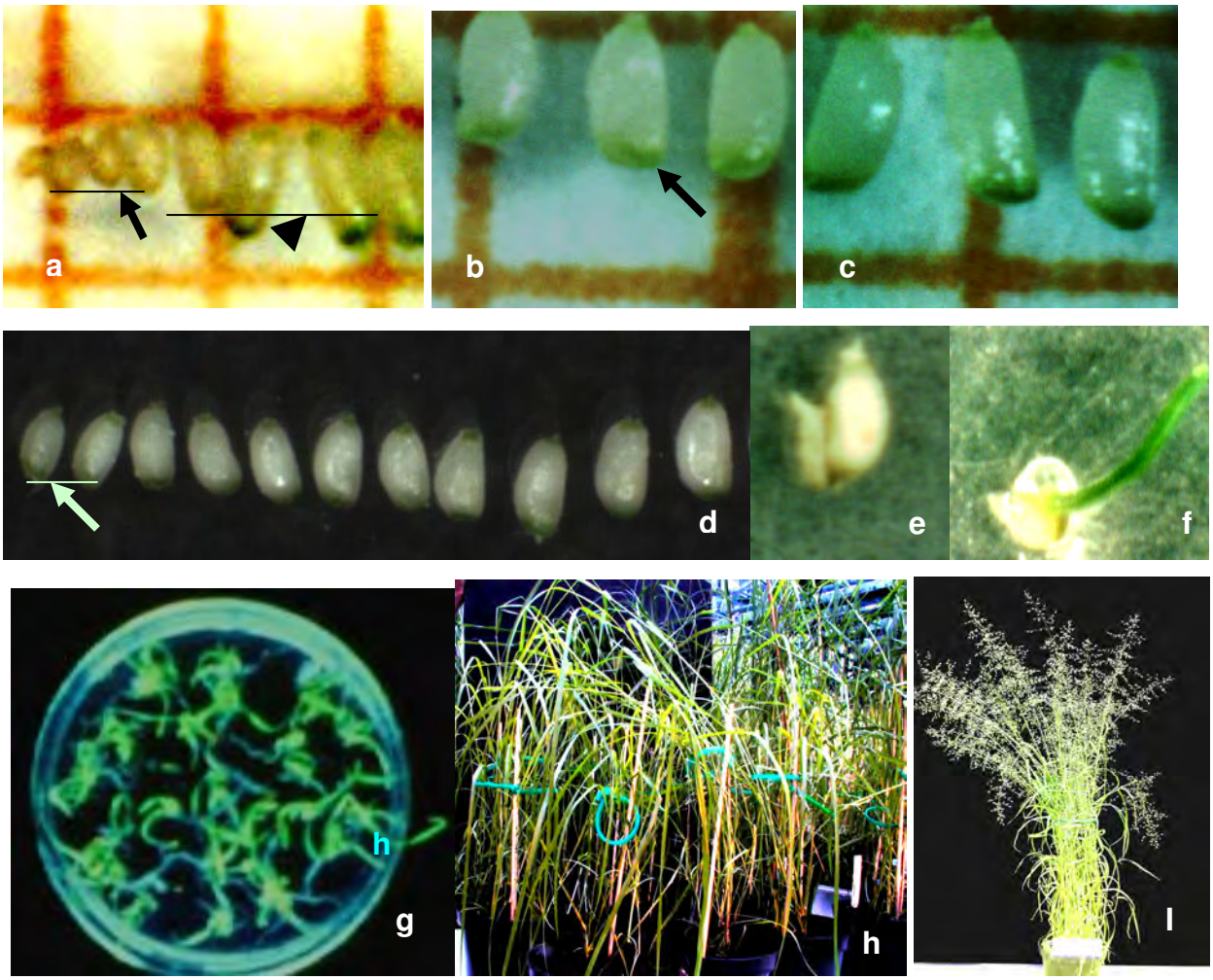

Fig. 3. The developmental stage of tef caryopsis used in ovary culture and the rescued germinated plants. **a)** early developmental stages of ovaries; ovary before pollination (far left), few hours after pollination (arrow), 1-2 dap (arrow head) **b)** 3-5 dap, embryo position in side the ovary (arrow) **c)** 6-7 dap **d**) 8-9 dap (arrow) and the subsequent growth of caryopsis **e)** embryo emerged out of the ovary in culture at maturity **f)** germination of the cultured ovary from the emerged embryo **g)** efficient germination of ovaries cultured in zero glutamine medium and transferred to the germination medium supplemented with GA_3 **h)** normal growth of plants derived from both explant types at the green house condition **i)** *E. mexicana* plant derived from embryo culture. (Magnification for Figs **a – g = X 10)**

Various factors which contributed to the successful rescue of the immature explants were evaluated and the results of each experiment are given below.

Experiment 1. Effect of Glutamine

Results in Tables 5, 6, 7 and 8 show the growth and development of explants of all genotypes studied in the various glutamine concentrations. Rescue of both explant types (Table 5) at various stages of growth (Table 6) of the tested genotypes (Table 7), decreased with the addition of glutamine to K99 medium. Besides, variation was highly significant (P<0.001) among the tested treatments (Table 5, 6 & 7) and the interaction between varieties and treatments (Table 8)**.** However, the very small embryos (20 µm) at the globular stage (Fig. 4a arrow) did not respond at the lower level of glutamine level (0-1 mM) and developed to early embryo and early coleoptilar stage (Fig. 4e arrow) at higher (3 & 6 mM) glutamine concentrations. However, these were unable to further develop and sustained longer and later died. Sub culturing to a fresh media did not help the growth of these embryos.

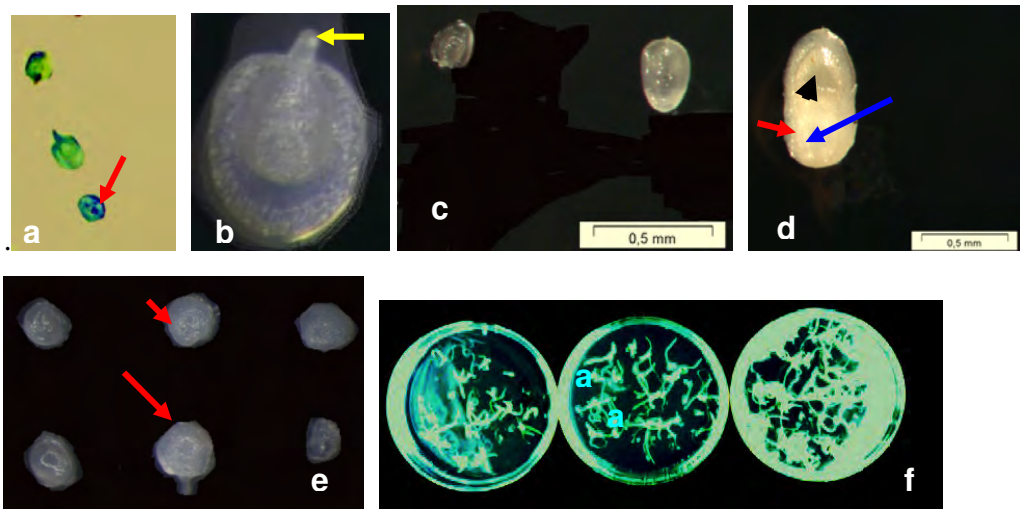

Fig. 4 Developmental stages of immature embryos of tef used for the embryo rescue experiment. **a)** very small embryos (20-30 µm, X40) **b)** small embryo (50 µm, X40) showing suspensor tissue (arrow) **c)** intermediate, 150 µm (left) and large 350 µm (right) embryos **d)** matured embryo indicating scutellum (short arrow) coleoptile (arrow head) and radical (long arrow) **e)** development of very immature embryos to globular stage (short arrow) and to early coleoptilar stage (long arrow) in culture, X40 **f)** germinating embryos in 0 mM (right), 1 mM (middle) and 3 mM glutamine (left), X10)

As Table 5 and 6 indicated, variation was significant among the tested treatments (0-10 mM) and variety x treatment interaction and not for explants and varieties (Table 6). Both ovaries and

embryos of variety DZ-01-196 decreased their success of rescue significantly with the increase of glutamine. However, the ovaries of the land race, Fesho, increased its percentage of germination at 1 mM glutamine level (Table 5, figure in parenthesis).

Table 5. Total number of ovaries and embryos (N), mean ± standard deviation of germinating plants of varieties, DZ-01-196 and Fesho with various levels of glutamine, transferred to K4NB, without GA_3 (Pooled data of three independent experiments)

	Ovary		Embryo	
Glutamine (mM)	N	Means ± SD	N	Means ± SD
0	150	64.5±0.12a (47.2±0.24b)	120	67.5±0.31a (62.9±0.32a)
1	130	48.5±0.21b (57.6±0.51a)	115	62.7±0.13a (61.3±0.19a)
3	145	43.9±0.06b (33.7±0.17c)	125	62.7±0.04a (50.6±0.34b)
6	150	43.5±0.31b (33 5±0.34c)	110	56.5±0.49b (47.2±0.38b)
10	135	29.7±0.50c (21.8±0.22d)	130	32.6±0.71c (29.3±0.43c)

N.B. figures in parenthesis are for variety Fesho. Means followed by the same letters are not significantly different at the 5% level of probability.

According to Table 5, the percent of germination of DZ-01-196 ranged between 29.7 to 64.5 % and 32.6 to 67.5 % for ovary and embryo cultures, respectively from the highest to the lowest treatments of glutamine in germination medium (GM) devoid of GA_3.

Table 6. Analysis of Variance for effect of glutamine on ovary/embryo rescue of three tef varieties-that were-germinated on medium supplemented with GA_3.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Variety	2	15.517	7.758	1.333	0.269
Explant	1	29.008	29.008	4.985	0.028
Treatment	4	4504.883	1126.221	193.527	<0.001

Variety x Treatment	8	290.817	36.352	6.247	<0.001
Variety x Explant x Treatment	8	72.883	9.110	1.566	0.146
Residual	90	523.750	5.819		
Total	119	5478.592	46.039		

Since variations among the first three levels (0, 1 and 3 mM) were insignificant at the first attempts for the embryo explant of variety DZ-01-196 (Table 5), experiments to follow were carried out using these three levels (1-3 mM glutamine concentration) in order to differentiate the appropriate glutamine level.

Table 7. Effect of ovary age and response of genotypes, (cultured on three levels of glutamine) on percent of germination and result calculated in Mean ± SD. Explants germinated on medium supplemented with GA_3. (Pooled data of 75 explants per each treatment).

Age of Ovaries →	**3-5 dap**			**6-7 dap**			**8-9 dap**		
Glutamine mM →	**0**	**1**	**3**	**0**	**1**		**0**	**1**	
↓ Varieties	Mean ± SD			Mean ± SD			Mean ± SD		
DZ-01-196	**98.3**±0.17a	85.0±0.31b	44.8±0.31c	90.0±0.65a	85.4±0.61a	36.2±3.15b	31.3 ±2.67a	20.3±1.77b	21.0±1.46b
DZ-CR-37	85.1±0.74a	39.4±2.76b	30.5±2.01c	79.1±0.43a	45.6 ±2.16c	58.3±1.37b	27.2±1.55a	18.1±3.17b	11.1±3.10b
Fesho	55.4±0.11a	45.6± 0.99b	35.0±1.87c	53.9± 1.26b	75.0±0.77a	13.2±5.11c	21.3±2.97a	13.7± 2.84b	9.3±4.01 c

Means followed by the same letter are not significantly different at 5% level of probability at each row in each column.

Table 8 Effect of embryo age and genotypes (cultured on three levels of glutamine) on percent of germination calculated Mean ± SD. Explants germinated on medium, supplemented with GA_3 (Pooled data of 75 explants per each treatment).

Age →	**5-6 dap**			**7-9 dap**			**10-12 dap**		
Glutamine (mM) →	**0**	**1**	**3**	**0**	**1**	**3**	**0**	**1**	**3**

Varieties ↓	% of germination	Mean± SD			Mean± SD		
DZ-01-196	0.0 0.0 0.7	91.5±0.91a	56.0±1.37b	32.0±4.21c	**99.3**±0.75a	56.0±1.73b	22.0±2.22c
DZ-CR-37	0.0 0.0 0.3	83. 7±0.64a	40.1±0.86b	26.5±1.81c	91.1±0.11a	41.3±2.11b	16.6±3.33c
Fesho	0.0 0.0 0.1	73.5±0.76a	76.6±0.59a	21.3±4.62b	85.4±0.69a	40.6 ±3.09b	15.5±5.12c

Means followed by the same letter are not significantly different at 5 % level of probability at each row in each column.

As Tables 8 and 7 show the highest rescues (99.3 and 98.3 %) were obtained from the oldest embryo ages and younger ovaries respectively from variety DZ-01-196 followed by variety DZ-CR-37 (91.1 and 85.1 %) all at zero glutamine level. However, ovaries of variety Fesho showed high germination capacity at 1 mM glutamine level. Despite of this, plants germinated in above 1 mM glutamine were wrinkled in appearance compared to plants germinated on zero glutamine.

In this study, the morphological variations among the germinated plants were evaluated by counting roots and tillers number and root and plant length of the two ovary growth stages grown in the two levels of glutamine (0 and 1mM). Significant differences were observed only in the root length at 5 % probability level and zero glutamine gave the best result (Table 9). This might implies that the presence of glutamine inhibited the root growth of the germinating plants and it seemed that root length is highly influenced by glutamine. However, there was no visual difference in morphology and fertility of the greenhouse grown plants.

Table 9. Average number of leaves and tiller and length of plant and roots of the plants derived from the two stages of ovaries grown under 0 and 1 mM glutamine levels.

Glutamine (mM) ↓	Ovaries Age (dap)	Number of leaves	Number of tillers	Root length (cm)	Plant length (cm)
0	3-5	3	1.0	19.8±0.33a	19.8±0.71a
	6-7	3	1.15	18.95±0.2a	22.95±0.65a
1	3-5	3	1.0	12.3±0.58b	18.9±0.01a
	6-7	3	1.1	12.2±0.92b	19.8±0.82a

Means followed by the same letter are not significantly different at 5% level of probability

Experiment 2. Effect of maltose

Ovaries and embryos were cultured in the absence of glutamine at the age of 3-5 dap and 6-7 dap respectively for this experiment. The ANOVA analysis (Table 10) was performed for variety DZ-01-196 and percent of germination were calculated for variety DZ-CR-37 using intermediate sized embryos at 0 and 1 mM glutamine (Table 11) levels. The result (Table 10) indicated that the variation among treatments were highly significant at $P<0.001$, but not for varieties, and the interaction between the two (varieties x treatments). The maltose level had similar effect for both explants that, the increase or decrease of the level of maltose above the optimum (250 mM) reduced the percent of rescued explants for variety DZ-CR-37 (Table 11). Besides, at lower concentration of maltose, most of the cultured ovaries showed precautious germination to shoots or roots and at the highest level to abnormally twisted leaves.

Table 10. Analysis of Variance Table on the effect of maltose level of two varieties (DZ-01-196 and DZ-CR-37) germinated on GM supplemented with GA_3.

Source of Variation	DF	SS	MS	F	P
Varieties	1	2.000	2.000	0.735	0.408
Treatment	2	469.778	234.889	86.286	<0.001
Varieties x Treatment	2	9.333	4.667	1.714	0.221
Residual	12	32.667	2.722		
Total	17	513.778	30.222		

Table 11. Effect of maltose levels at two concentration of glutamine on percent of germination of ovary and embryo culture for variety DZ-CR-37.

	Ovary					**Embryo**				
Glutamine mM	Maltose (mM)	N	% of normal germination	Shoot only	Root only	N	% of normal germination	Shoot only	Root only	Globular to sutellar stage only
0	150	75	22.2	41.6	19.4	75	44.4	-	-	42.9

0	250	75	86.3	-	-	75	90.7	-	-	-
0	350	75	11.4	50.0	25.0	75	6.1	-	-	56.7
1	150	75	32.8	48.2	21.0	75	10.0	-	-	30.8
1	250	75	43.3	-		75	41.7	-	-	-
1	350	75	14.8	61.0	17.9	75	15.0	-	-	24.2

Experiment 3. Effect of duration in dark culture

Treatments: 0, 3, 5, 7, 10, 12, 15, 20, 25, and 30 dark culture days were compared before transfer of the grown explants to light chamber for germination. A total of 60 explants were used for each treatment. None of the directly cultured (0 day) explants at light was able to precede development. During the culture of very immature zygotic ovaries (1-3d), the presence of embryos was not observed under the binocular microscope because the zygotes at this stage are at cell differentiation or pro-globular stages (8-16 cells). However, few days after dark culture, the presence and growth of embryos inside the ovary became visible and started to emerge gradually out of the ovary body (Fig. 3e)**.** After maturation of the cultured explants, they were transferred to the germination medium (GM) and cultured under light chambers. Proper growth and normal germination of roots and shoots (Fig. 3f) from the *in vitro* matured ovaries and embryos were observed after 2-3 days of transfer to light.

There were a significant variations ($P<0.01$) among the treatments (see Tables 12 and 13) for both explant types. Ovaries showed faster (Fig 5) and higher germination when they were cultured only for 5 days in dark whereas, the intermediate sized immature embryos required a minimum of 15 days of dark culture period before transferred to light.

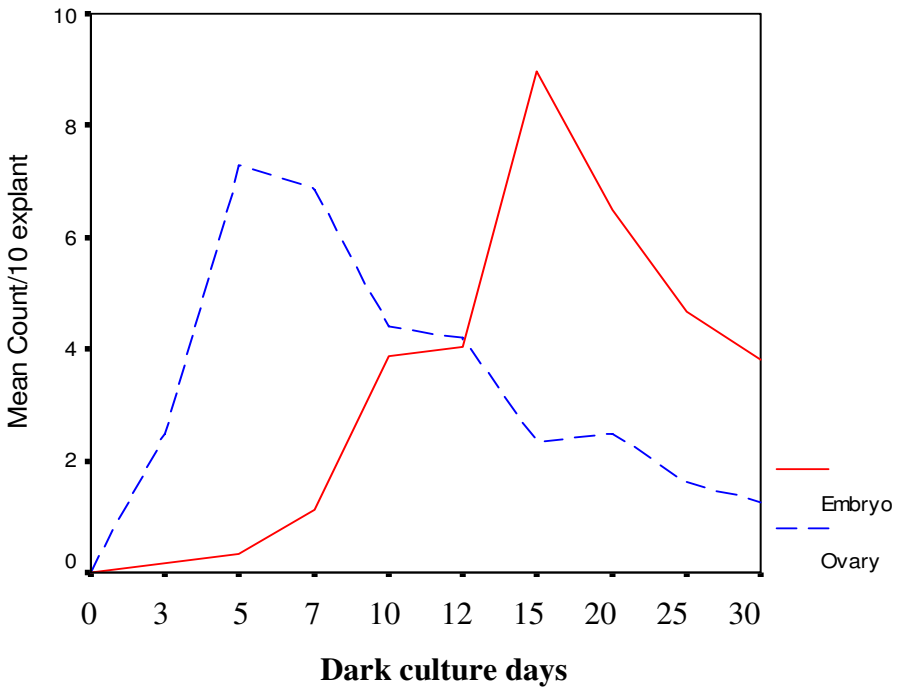

Fig. 5 Effect of dark culture days on the germination of ovaries and embryos.

Table 12. Analysis of Variance Table on the effect of dark culture duration in embryo rescue.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	9	3.18717494	0.35413055	31.77	<.0001
Error	30	0.33442912	0.01114764		
Corrected Total	39	3.52160406			

C.V. = 30.5

Table 13 Analysis of Variance Table on the effect of dark culture duration in ovary rescue.

Source	DF	Squares	Mean Square	F Value	Pr > F
Treatment	9	2.02901909	0.22544657	26.02	<.0001
Error	30	0.25994977	0.00866499		
Corrected Total	39	2.28896887			

C.V. = 28.2

The highest mean germination (99%) (Table 8) was generally obtained from the embryos than that of the ovaries (Table 7). However, the development and germination period depended on the age of embryos. Matured larger embryos (Fig. 4d) germinated after few days of culture in the

induction medium. However, these stages were not used for rescue purposes due to their mature size.

Delayed transfer of ovaries from the dark culture to the germination medium reduced the ability of the emerged embryos with the emergence of mostly roots or shoots. Similarly transfer of premature embryos to light led to stunted growth and abnormal germination. Therefore, the successful development of the explants during culture initiation was based on the ages and dark culture duration.

Experiment 4. Effect of types of explant (isolated ovaries and embryos)

In contrast to the isolated very immature embryos, the youngest ovaries (3-5dap) grew faster and required only a total of 15 days of *in vitro* culture before transplanting to soil. However, the cultured isolated small embryos required more than four weeks of *in vitro* culture to transplant to soil. In general, as far as the rescue is concerned, the culturing of ovaries was simple compared to embryos because no labour is required for the excision of the small seized embryos.

Experiment 5. Effect of age of explants

From the preliminary observation trial it was clear that an attempt to rescue the very young stage, (0-2 dap) ovaries and isolated ovules, using the various levels of glutamine in the given medium was not successful. The majority of ovaries isolated at this stage were shrinked within few days of culture while, the number of growing ovaries increased when isolation was made after 3 days of pollination for all genotypes including the wild species. Therefore, ovaries of 3-5, 6-7 & 8-9 and embryos at 5-6, 7-9 and 10-12 dap were compared in the three glutamine levels (0, 1 and 3 mM) using the three varieties (Tables7 and 8).

The two improved varieties (DZ-01-196 and DZ-CR-37) responded best when using the youngest age ovaries and for the land race, Fesho, when using the intermediate age, 6-7 dap ovaries. The germination percent dramatically dropped for the oldest age ovaries, 8-9 dap (Table 7) and the youngest, 5-6 dap embryos (Table 8). The difference in germination percent between the cultured isolated embryos of 7-9 dap and the same age ovaries, which contained un excised embryos, was high. Isolated embryos have shown higher germination than ovaries for all genotypes. Matured embryos (10-12 dap) during excision started germination within few days in

culture and most of the oldest ovaries remained as a wrinkled seed and turned browny without germination. Since, these growth stages are too old for hybrid embryo rescue after interspecific crosses; young ovaries can be taken as the most convenient materials to rescue hybrids before abortion takes place.

Embryos from pro-globular and globular stages during isolation (5-6 dap and approximately 20-50 µm) usually did not respond to the treatments studied and terminated growth. These embryos were sustained for two weeks in culture and remained unchanged for longer period. Further culture of these embryos to fresh medium and sub culturing in every one week interval did not help. These differences in germination between the cultured isolated embryos at the age of 7-9 dap and the same age embryos cultured inside the ovary (ovary culture) was not clear. In all genotypes, the isolated embryos showed higher germination than ovaries cultured. Therefore, the success and development of the explants was based on the type and ages of explant during culture initiation.

Experiment 6. Effect of GA_3 in germination medium (GM)

As shown in Table 14 the percent germination of explants was highly improved by the addition of 1 mg/l GA_3 in the germination medium. High, 98.2 % embryo and ovary rescue was obtained from DZ-01-196 (Fig.3g) followed by DZ-CR-37 (95.7 %) and Fesho (78.6 %) respectively, the later by using 1 mM glutamine. The percent of germinating explants increased approximately 1.5 fold only by adding GA_3 in the GM.

Table 14. Percent germination on GM containing GA_3 of the cultured immature embryos (N) of the three tef varieties using three levels of glutamine.

Treatment →	**GM without GA_3**				**GM supplemented with GA_3**			
Genotypes ↓	N	Glutamine (mM)			N	Glutamine (mM)		
		0	1	3		0	1	3

DZ-01-196	120	67.5	62.7	42.7	150	98.2	86.7	62.1
DZ-CR-37	115	63.7	60.0	36.5	150	95.7	77.8	48.9
Fesho	125	52.9	61.3	50.6	150	69.6	78.6	58.6

Experiment 7. Response of genotypes including wild species

In all experiments, DZ-01-196 performed best compared to the other tested genotypes. For the wild species, both explant types were rescued using the intermediate ages of the explants without glutamine and germinated on GM containing $GA_{3.}$

Variation among the wild species was high and the highest percent of germination (94.3 and 92.4 %) was obtained from *E.mexicana* followed by *E. pilosa* with 82.4 and 76.3 % for embryos and ovaries rescue respectively (Table 15). *E. papposa* was the least and its seeds had shown dormancy when directly sown in the soil. However, the dormancy was broken down by germinating the seeds in K99ER medium and explants were taken from these plants.

Table 15. Percent of rescued ovaries and embryos of *E.pilosa, E. papposa* and *E.mexicana* in K99ER medium without glutamine in GM containing $GA_{3.}$

Species ↓	Ovary		Embryo	
	N	%	N	%
E.pilosa	100	76.3	100	82.4
E.mexicana	100	92.4	100	94.3
E.papposa	100	32.7	100	52.7

3.2.3 *In vitro* crossing and *in vivo* wide hybridization:

In vitro crossing techniques of both inter and intraspecific hybridization is as tedious as *in vivo* pollination. On the other hand wide crossing of tef with maize did not require much effort because pollen grains of maize can easily be dispersed on tef stigma in *in vitro* crossing but it is relatively easier at *in vivo* condition. Successful *in vitro* fertilization was observed with intervarietal crosses (Table 16)**.** In most crosses the pollinated pistils were shrunken 6-12 hours after pollination indicating self incompatibility between two species and those belonging to the

different genera. It was impossible to detect whether this incompatibility took place after pre or post fertilization. The clear observation was that, there was no embryo formed to isolate for the rescue. Similarly the successful pollination of the intraspecific crosses between variety, DZ-01-196 (mother plant) with the freshly dehisced pollen of *E.mexicana* and *E.papposa* was also ended with a similar result (shrinking) but, here the pistils were collapsed after some enlargement. In the latter it seems that fertilization had taken place between the two species and the collapse of ovary may take place after post fertilization. Despite of these, one ovary out of 50 crosses of *E, tef* with *E.papposa* was developed and the immature caryopsis was successfully rescued using the established protocol and the plantlet was transferred to soil but this progeny died few days after transfer to the greenhouse. In general, all the transplanted plants derived from the ovary and embryo rescue of all tef (Fig. 3h) and wild species, *E.mexicana*, (Fig. 3i) were vigorous and normal in both morphology and fertility status.

Table 16 In vitro crosses between tef and its wild relatives including wide crossing.

Genotypes ♀ ♂	Total No. of pistils	Collapsed No. of pistils	Survived No. of ovaries	No of hybrids	Transplanted Hybrids	Hybrids grown to maturity
DZ- CR-37 x DZ- 01-196	50	38	12	12	6	6 grown
DZ-01-196 x DZ- CR -37	50	40	10	8	6	6 grown
DZ-01-196 x *E.mexicana*	50	50	0	0	0	Pistils collapsed
DZ-01-196 x *E. papposa*	50	44	6	1	1	Early death, few days after transf
DZ-01-196 x maize	100	100	0	0	0	Pistils collapsed
DZ-01-196 x wheat	50	45	5	0	0	Pistils collapsed

3.3 Discussion

Isolation of the minute embryos of tef out of the immature caryopsis is very difficult particularly for inexperienced person. In tef, flowering commences acropetally in spikelet basis and basipetal in panicle basis (Berhe, 1975; Ketema, 1983). While the bottom florets of a spikelet set seed the

top florets do not commence anthesis. Therefore, one can find young embryos and ovaries on the mid-top position while the bottom two-three florets were getting older for isolation.

3.3.1 Factors affecting embryo rescue

3.3.1.1 Culture medium: From the preliminary observation trial, except K99 medium, both N6 and MS were not found to be suitable for the development of the immature tef explants. For instance, in a preliminary observation trial, the responses of the cultured matured seed explants of tef to K99 and modified MS media had shown distinct variation for callus induction and somatic embryo formation, in that only non-embryonic amorphous callus was obtained from the MS while embryonic callus were obtained from K99. Therefore, K99ER (K99 for embryo rescue) was used solely as embryo rescue culture medium for both explant types. This medium has also been used for wheat embryo culture in the same laboratory (Kumlehn, personal communication). Gu et. al., 1990 studied the effects of various factors affecting immature embryo culture of barley and explained that medium composition plays the major role compared to size of explant and genotypes. The most widely used media in embryo culture are MS (Murashige and Skoog, 1962), N6 (Chu, 1978) and Gamborge B5 (Gamborge et al., 1968) with certain modification. In many cases reducing the levels of inorganic salts (except potassium and calcium) such as ammonium (NH_4NO_3) and Fe-EDTA to approximately one-half of MS medium and also exchanging KCI for KNO_3 are used for effective embryo culture thus increasing the survival rates of small excised embryos (Monnier, 1976 and 1980). K99 medium is constructed with low inorganic salts compared to both MS and N6 medium and is supplemented with maltose instead of sucrose. This medium may contain suitable nutrient source when it is compared to MS and N6 for the growth of immature explants of tef and other *Eragrostis* species.

3.3.1.2 Maltose: Carbohydrates play an important role in maintaining suitable osmolarity and the osmolarity requirement differs according to the stage of growth of the embryos. For instance, the younger the embryos the higher is the medium osmolarity required (Hu and Wang, 1986). Rietsema et al., (1953) observed this in Datura embryos and as they stated, matured embryos grew even without sucrose. Similarly, in the present study, the higher the maltose level the lower was the germination capacity of the cultured explants (both types). At the lower maltose concentration precautious germination of the cultured immature embryos were observed leading

to weak and stunted growth displaying only those structures already present during the time of culture without any germination. At the highest level (350 mM) normally embryos became thick and browny and failed to germinate. So the maltose level at 250 mM was found to be suitable to both immature explant types of all genotypes. The cause of precautious germination and its control have been reviewed by Norstog, (1972, 1979) in barley. Norstog, (1979) summarized factors in the culture medium that contribute to suppress precautious germination and these include high osmotic pressure, elevated potassium and ample nitrogen source in the form of ammonium salts, organic acid, and ABA.

3.3.1.3 Amino acids: The level and balance of inorganic nitrogen ions in the form of ammonium or nitrate and the use of organic sources such as malate, citrate or amino acids may be critical in some species. Among the various amino acids and their amides, adenine and glutamine were the most effective ones next to asparagine and frequently used to stimulate *in vitro* embryogenesis (Hu and Wang, 1986). For instance for barley (Norstog and Smith, 1963, Cameron-Mills and Duffus, 1977), *phaseolus* (Mok et al., 1978) and Datura (Paris et al., 1953, Motsubara, 1964) glutamine was used in the immature explant cultures. Glutamine with a combination of a small concentration (0.05-1.0 mg/l) of casein hydrolysate (CH) was also effective to control precautious germination. In this study however, the negative effect of glutamine on the cultured immature, ovaries and embryos is not clear. However, it may be due to several factors that, immature explants at this stage may store enough amounts of endogenous amino acids including glutamine and require only a supply of energy (sugar) for normal differentiation and development of zygotic embryogenesis in an optimized media like K99ER. The other reason could be synthesis of endogenous amino acids may continue *in vitro* during the culture period and this extra glutamine may not be required. It is known that tef has a protein content of (11 %) and contained most of the important amino acids, glutamic acid being the dominant (Bekele, 1995). However, the extremely small embryos, below 30 µm failed to properly develop, mature and germinate even when provided with low level of glutamine containing medium. This might indicate that the limiting factor for the growth of these sizes of embryos may not be only the glutamine, but other amino acid sources like asparagines and casein hydrolisate (CH).

According to Hu and Wang (1986), embryos excised from the developing seed at or near the mature stage are completely autotrophic, germinate and grow on a simple inorganic medium with a supplemented energy source. However, for immature explants/embryos progressive change of nutritional requirements are expected during the development of excised immature embryos (Raghawan, 1965). The failure of the excised immature embryos of tef at globular to pre scutelar stages, and ovaries at 1-2 dap could be due to similar reasons. Monnier (1976) had designed an approach to rescue capsella immature embryo. In such an approach assurance of a continual gradient in the medium composition over time via diffusion for the excised embryos cultured in two media of different compositions, where the high osmolarity medium surrounded by low osmolarity medium to let the cultured capsella embryos to develop and germinate. This attempt had not yet been tested in tef embryo culture condition.

3.3.1.4 Growth regulators: Although growth regulators are not normally required for zygotic embryo culture, exogenous hormones such as cytokinin, auxin and or gibberellins (ABA, IAA and GA) may stimulate the development and germination of the extremely small embryos in culture. In tef the presence of auxin, 2, 4-D at a low concentration (0.1 μM) in K99 medium, triggered the induction of small calli which induced somatic embryos and regeneration (data not shown). Even the small embryos (30-50 μm) were able to induce somatic embryos with the addition of 2,4-D. Somatic embryogenesis is the other alternative approach for zygotic embryogenesis in embryo rescue. This indirect approach was studied in tef (see Chapter 4). Raghavan (1965) also obtained plants when capsella's late globular stage embryos were cultured with the addition of hormones in the culture medium. However, the medium requirement for early globular stage was unknown. Generally, refinement of the medium composition that can sustain the growth and development of those embryos by using feeder cell approach to the extremely small embryos of tef and ovaries of 0-2 dap is required. Moreover, somatic embryogenesis is the other alternative approach for zygotic embryogenesis which can be used for embryo rescue.

3.3.1.5 Age and type of explant: The other important factors, which determine the success of embryo culture is the age of the embryos. The more advanced embryos are less complexes in their nutritional requirements. On the other hand, there is a decrease in the percent of cultured

ovaries remaining viable as their age progress (Hu and Zenettine, 1995). This is clearly in agreement with what was observed in the older age of ovaries in tef. The older ovaries remained mainly dormant in the medium used and gave low germination percent. In the inter-specific hybridization of the incompatible species, embryo abortion due to embryo-endosperm incompatibility of the parents is the main obstacle to obtain hybrid seeds. Therefore, refined embryo/ovary rescue technique should be developed to prevent the drawbacks of abortion. For this purpose selecting the appropriate ages of embryo/ovary and appropriate explant type (embryo or ovary) should be the main target.

Whenever embryos are difficult to excise or when the success of rescue is very low, the other alternative approach is ovule and/or ovary culture, especially to rescue the extremely young embryos (Van Tuyl et al., 1991). The hybrid embryo may directly germinate from the ovule as tef immature ovary culture or can be dissected out after a period of in ovulo growth to be followed by embryo culture. In brassica (Ross, 1980) small ovaries can be cultured in their entirety, while large ovaries as in lillium (Hai-Shan, 2002**),** usually are cut in section and cultured.

The other reason for the failure of the excised very small pre-early globular embryos in culture could probably be due to the lack of suspensor tissue during the culture initiation. Suspensors are known for the up-take of nutrient to the growing embryo from the medium. This is true because early embryos, which contain suspensor tissues survived, matured and germinated normally when transferred to the germination media. Suspensor may also play an important role in embryo development by acting as a source of hormones to the young developing embryo and it might also take over the function of endosperm as mentioned by Czapik and Izmailow (2001) in *Phaseolus coccineus.*

Culturing the immature ovaries instead of tef embryos is advantageous except for the extra work and time needed. It is thus possible to rescue the very immature stage hybrids which other wise will be impossible in embryo culture in the given medium. Besides, as suggested by Kanoh et. al., (1988) the fast growth or short period requirement of the cultured ovaries/immature caryopsis compared to the excised immature embryos could be due to the presence of endosperm in the former explant. Kanoh et. al., (1988) also suggested that the cultured ovaries have an advantage by receiving some nutrients and hormones from the ovary wall, placenta and endosperm tissue.

These tissues may supply nutrients or hormones not available in the culture medium, but could stimulate embryo germination. It is known that the endosperm is the tissue that nourishes the young embryo from fertilisation to the time until the green leaves become functional. However, it has been demonstrated that both embryos and endosperm are able to self-organize without the mother tissue in a manner similar to that of *in vivo* (Kranz and Kumlehn, 1999). In culture the zygote without an endosperm and an endosperm without an embryo developed to what is observed *in vivo* (Kranz and Kumlehn, 1999).

According to Fischer-Iglesias and Neuhaus (2001), immature seeds are the first higher plant source of most of the known plant hormones and the major auxin found is IAA, which increased during embryo development in wheat reaching to about 0.4 µM at the fully differentiated immature embryo stages. Besides, reproductive structures of plants are not only rich in cytokinines but also contain a great diversity of these compounds. This could be one of the reasons for the fast development of the cultured ovaries, which nourishes the developing embryos inside the ovary, placenta and endosperm tissues. Addition of similar growth hormones, available in the endosperm might improve the rescue of isolated immature embryos. Kumlehn et al., (1998) and Kumlehn and Kranz (1997) provided normal embryos that germinated from wheat ovules and zygotes excised 3-6 hours after *in vivo* pollination. The explants were cultured in medium having feeder cells like endosperm tissues of maize and a week old sporophytic cells of barley derived from microspore as a nourishing agent, respectively. High efficiency cell division and embryo formation resembling in situ embryos were obtained and these embryos were germinated to normal and fertile plants. Similarly, Hu and Wang (1986) showed the transfer of the excised small, 20 µm embryos of barley into wheat endosperm tissues and these embryos cultured in the nutrient medium showed normal development and germination.

The observed low efficiency germination of embryos from the older ovaries could be due to the fact that the composition of the cultured medium (K99) was beyond the requirement of the developing embryo. In contrast, according to Hu and Wang (1996) and Hu and Zanettini (1995) as progressively younger embryos are excised the medium requirement becomes progressively complex to permit the expression of the total development of the embryo (Hu and Wang, 1996).

3.3.1.6 Germination medium: Addition of 1 mg/l GA_3 in the germination medium improved the germination of embryos by 1.5 percent compared to the control. Schooler (1960) showed the

positive effect of gibberellic acid in embryo culture media using *Hordium vulgare.* Immature seeds are rich sources of gibberlic acids (GAs) and these are high at early to mid embryo development (Fischer-Iglesias and Neuhaus, 2001). GA_3 also improved the germination of the dormant matured ovaries of tef. It is known that gibberlic acid breaks dormancy in seeds.

In general, the result of the present study showed a successful development of rescue technique for embryos and ovaries culture of tef and its relatives for the first time. By using this protocol embryo/ovary culture following *in vivo* pollination can facilitate interspecific hybridization of tef with its wild relatives.

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Chapter 4

High efficiency somatic embryogenesis and plant regeneration from immature embryos of tef

4.1 Materials and Methods

Plant materials: Three tef varieties, DZ-01-196, DZ-CR-37 and the land race Fesho were grown in greenhouses at the Institute of Biozentrum, Klein Flottbek und Botanischer Garten (AMPII) and Institute fur Pflanzengenetik und kulturepflanzenforschung (IPK) Gatersleben, Germany.

The growth condition and sterilization of panicle is as described in Chapter 3. Panicles containing immature caryopsis (Fig. 6a), 7-10 days post anthesis were used for this experiment.

4.1.1 Isolation of immature embryo: Immature zygotic embryo isolation is as described in Chapter 3.

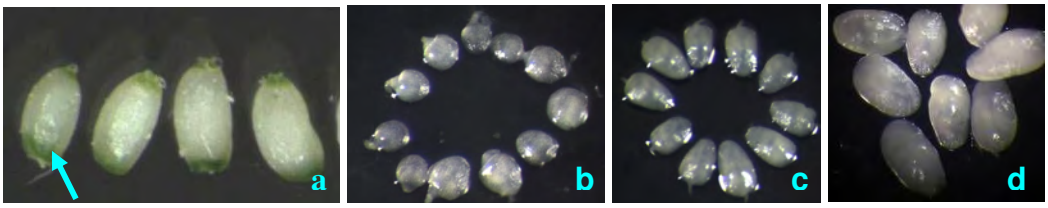

Fig. 6 Explants used in somatic embryogenesis experiment**. a)** Immature caryopsis (chalazal end, embryo side, (arrow) **b)** small (S) sizes 50-100 µm, (50 X) **c)** intermediate (I) size 101-350 µm (40 X) d) large (L) size 351-750 µm (30 X)

4.1.2 Culture condition: Ten to fifteen embryos per dish were placed on the media with the scutellum side up. Embryos were cultured according to their initial size; small (S), 50-100 µm, intermediate (I), 101-350 µm and large (L) 351-750 µm (Figs. 6b, c & d respectively) isolated from various kernels of the same sized spikelets, separately in each experiments. 35 mm petri dishes were filled with 3 ml of induction medium and the embryos were plated as the scutellum side up. The petri dishes were sealed and cultured in the dark at 26 ±1°C for 2-4 weeks depending on the size of the explant, genotype and medium. At least 60 embryos were tested in each experiment.

4.1.3 Media combinations: In the preliminary observation trial K99 medium (Table 4) was compared with modified L3 (Jähne et. al., 1991), N6 (Chu, 1978), and MS (Murashige and Skooge, 1962) media to culture the immature embryos of tef. Latter based on the preliminary result K99 was chosen to study the effect of various levels of media components such as glutamine, maltose, 2, 4-dichlorophenoxyacetic acid (2, 4-D) and phytagel by mostly using variety, DZ-01-196. Initially various concentrations (0.2 0.3, 0.4, 0.5 and 0.6 %) of phytagel were compared by supplementing the medium with 7 mM glutamine, 10 µM 2,4-D and 250 mM maltose. The other medium components were later optimized by using the various concentrations

of each component in separate experiments. Each time the best concentration of the component was used to optimize the subsequent trials. Lastly, the effect of the mineral salts of the modified MS (Murashige and Skoog, 1962) medium was compared with the modified K99 by supplementing all the optimal combinations of K99 in both media using the three tef genotypes. MSSI (MS, shoot induction medium, having 0.1 µM 2, 4- D was compared with K4NB only in experiment 5.

4.1.4 Sub-culture condition: Only the non-embryogenic calli of variety Fesho were selectively sub cultured in the same fresh medium.

4.1.5 Regeneration medium and culture condition: Embryonic calli were transferred to regeneration medium, K4NB (see Table 4) and cultured at 16/8 h day/night light condition. To investigate single somatic embryos germination, mature somatic embryos were separated from the callus tissue and plated on medium devoid of growth hormones. In order to quantify the number of plantlets per explant, single samples from the highly regenerable calli were splited and subcultured in new fresh regeneration medium to observe the maximum capacity of the explant in total shoot production.

4.1.6 Data collection and analysis: Percent of callus induction, somatic embryo (SE) formation, shoot regeneration and average number of shoots per explant was calculated as the number of embryos showing plant regeneration out of the total number of plated embryos on the initial medium. CRD design was used and ANOVA Table was constructed to analyse the significant differences at or below 0.05 probability level using Duncan's method. The following independent experiments were carried out.

Experiment 1. Effect of glutamine levels: 0, 1, 3, 6 & 10 mM using 250 mM maltose, 0.4% phytagel and 10 µM 2, 4-D using varieties Fesho and DZ-01-196

Experiment 2. Effect of various levels of maltose: 150, 250 and 350 mM using 6 mM glutamine

Experiment 3. Effect of various levels of 2, 4-dichlorophenoxyacetic acid (2, 4-D): 0, 5, 10, 15, and 20 µM, using both varieties

Experiment 4. Effect of the various levels of phytagel: 0.2, 0.3, 0.4, 0.5 and 0.6 %

Experiment 5: Effect of media salts, (K99 was compared with the modified MS using varieties DZ-01-196, Fesho and DZ-CR-37)

4.2 Results

4.2.1 Embryogenesis and somatic embryo induction: Immature embryos of all varieties germinated in growth regulator-free media. However, 80-100 % callus induction and somatic embryo formation was exhibited in the 2, 4-D and glutamine containing media within the first two to three weeks of culture for all varieties tested. Although the size of calli at zero glutamine level was extremely small (< 3 mm), in general the sizes of the induced calli corresponded directly with the initial explant size and the culture medium used.

Two types of embryogenesis and three developmental phases were observed. The first phase was embryo expansion which occurs 1-3 days after culture (dac), where the scutellum expanded and became globular, these eventually structured to the second phase, embryo restructuring stage (Fig. 7a), which was mainly observed at the seventh day and continued to the third phase of induction of somatic embryos (SE) 14 dac from the edges of the growing embryo (Figs. 7b and c). This type of embryogenesis is regarded as direct, without a callus phase and is the main characteristic of the small and intermediate size embryos. The other type of embryogenesis is indirect embryogenesis appearing after a callus induction phase (Fig. 7d) and is followed by the latter emergence of somatic embryos from the induced compact embryonic calli. This is, mainly formed from larger sized embryos.

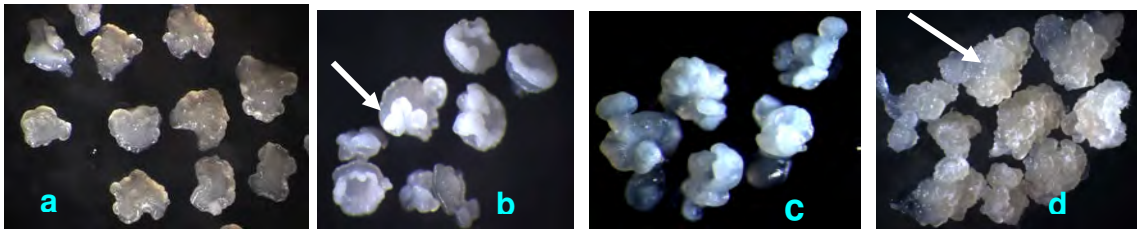

Figure. 7. Somatic embryogenesis from the cultured immature explants of tef. **a**) embryos after 7 days of culture, restructuring stage **b**) SE (arrow) formation after 10 days **c)** SE after 14 days **d)** SE after 21 days of culture from small, intermediate and large sized embryos respectively

4.2.2 Effect of embryo size: *Very small pro embryos below 50 µm, were usually non- responsive or form somatic embryos after a long cultre period. However, the smaller size embryos (100-150 µm) took only 4 weeks of* in vitro *culture to be transplanted to soil. That is*

duration of somatic embryo formation depended on the size of the explant. The smaller explants showed faster embryo formation. Only 10-12, 12-14 and 17- 21 days was required for small, intermediate and large sizes respectively (Figs. 6b, c, and d) for the two improved varieties to transfer to the regeneration medium (RM). In spite of this, the developmental phases of the cultured explants varied according to the composition of the media (See the results of each experiment). High quality somatic embryos were obtained after two to three weeks of culture. Although variation for SE formation and regeneration was observed among the three sizes, intermediate sizes out performed the rest (Fig. 8).

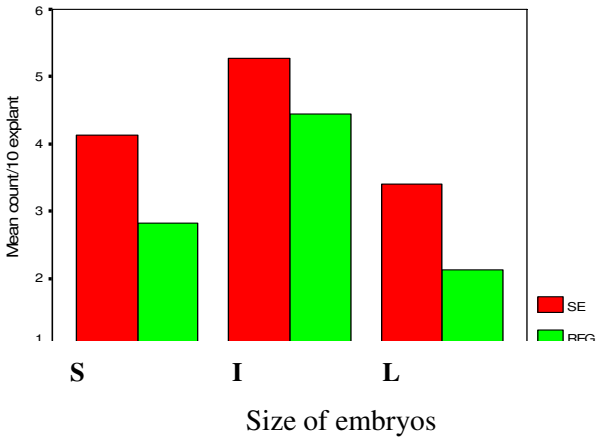

Fig. 8 Mean number of intermediate sized embryos cultured on 6 mM glutamine and 0.4 % phytagel and showing somatic embryos (SE) and regeneration using variety, DZ-01-196.

Experiment 1. Effect of Glutamine:

Embryogenesis increases with the increase of the concentration of glutamine up to the level of 6 mM for variety DZ-01-196 (Figs. 9a and b). Highly significant variation ($P< 0.01$) in the total number of somatic embryonic calli formation (Table 17b) and shoot regeneration (Table 17c) was observed between the tested treatment levels after 4 weeks of culture initiation. In the first two weeks, no explant on media containing 1 mM glutamine were able to form embryonic calli while calli on 3 & 6 mM glutamine containing media were ready to be transferred to regeneration medium (RM). None of the cultured explants were able to induce regenerable calli at 10 mM level (Fig 9b). The highest embryogenesis and regeneration was obtained at 6 mM followed by 3 mM for both varieties, DZ-01-196 and Fesho (Table 18) in the order of explant

size, intermediate, small and large (Fig. 9a). A maximum of 100 % somatic embryonic calli formation and 97.4 % regeneration were obtained at 6 mM glutamine for variety DZ-01-196 and 86 % embryonic calli and 75 % regeneration at the same concentration for variety Fesho (Table 18) when RM containing 0.2 µM of 2, 4-D was added for the later variety.

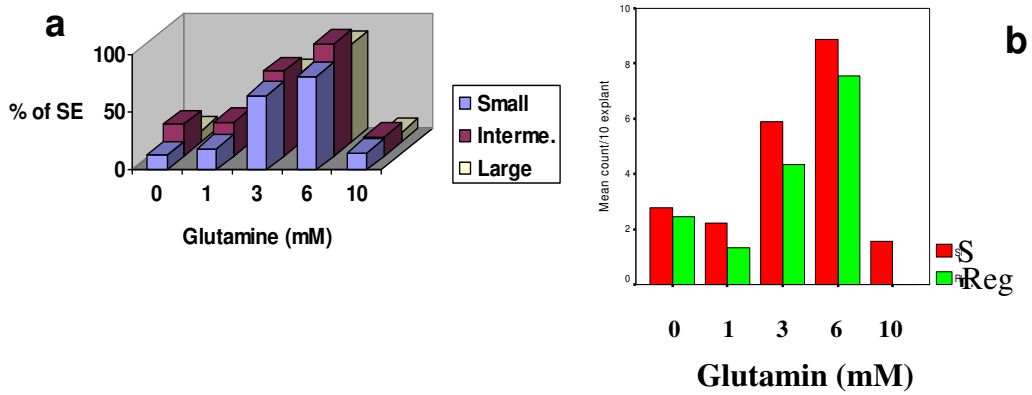

Fig. 9 Effect of glutamine levels on % somatic embryo (SE) formation and regeneration (Reg), variety DZ-01-196. **a)** Glutamine effect on somatic embryos formation using the various sizes of embryos **b)** glutamine effect on somatic embryo formation and regeneration from the intermediate sized embryos (data scored after four weeks of culture)

The distinct variation between the two genotypes was that zygotic embryos of Fesho required sub culturing after 2 week in order to form somatic embryos and took at least 4 weeks to transfer to RM. Up to 70 % increase in somatic embryo formation was observed by sub culturing. Therefore, variety Fesho was not used for further studies and DZ-01-196 at 6 mM glutamine was taken in the optimization of the other experiments.

Table 17 a, b, c. Two way ANOVA Table for the effect of glutamine on (a) callus induction (b) somatic embryo formation (c) plant regeneration using the three sizes of embryos of variety, DZ-01-196

a) Callus induction					
Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Treatment	4	287422	71856	5879	<0.001
Size	2	22533	11267	0.922	0.409
Treatment x Size	8	50578	6322	0.517	0.834
Residual	30	366667	12222		
Total	44	727200	16527		
b) Somatic embryogenesis					
Treatment	4	843867	210967	41.638	<0.001
Size	2	35244	17.622	3.478	0.044

Treatment x Size	8	41200	5.150	1.016	0.445
Residual	30	152000	5.067		
Total	44	1072311	24.371		
c) Regeneration					
Treatment	4	675.022	168.756	44.671	<0.001
Size	2	74.133	37.067	9.812	<0.001
Treatment x Size	8	166.311	20.789	5.503	<0.001
Residual	30	113.333	3.778		
Total	44	1028.800	23.382		

Table 18. Effect of glutamine in the somatic embryo and plant regeneration of two varieties

	Glutamine levels (mM)																	
Varieties	**0**				**1**				**3**				**6**				**10**			
DZ-01-196	Em No.	CF (%)	SE (%)	PR (%)	Em No.	CF (%)	SE (%)	PR (%)	Em No.	CF (%)	SE (%)	PR (%)	Em No.	CF (%)	SE (%)	PR (%)	Em No.	CF (%)	SE (%)	PR (%)
Small (S)	60	46.7	30.0	10.0	60	79.5	17.0	3.0	69	89.3	67.3	57.1	68	100	81.2	52.6	63	100	15.1	0.0
Interm, (I)	60	66.7	33.3	30.0	60	86.1	40.0	17.0	62	97.2	71.2	65.3	69	100	100	97.4	60	100	23.3	0.0
Large (L)	60	64.3	20.0	17.0	60	87.5	10.0	5.0	60	96.0	42.0	38.0	65	100	84.4	75.6	68	100	8.3	0.0
Fesho Small (S)	75	26.3	10.0	0.0	75	19.7	7.5	0.5	75	49.1	48.0	32.0	75	87.0	66.0	48.0	75	82	43.0	2.0
Interm, (I)	75	46.1	15.0	4.0	75	26.3	10.0	3.3	75	57.0	59.0	40.0	75	94.1	75.0	67.0	75	100	44.5	7.0
Large (L)	75	14.3	8.0	2.0	75	27.1	3.5	1.5	75	26.0	45.0	34.0	75	79.3	65.0	54.0	75	100	38.0	1.8

Em - zygotic embryos, CF -callus formation per zygotic embryos cultured, SE - somatic embryo formation per zygotic embryos cultured, PR, plant regeneration; Interm- intermediate,

Experiment 2. Effect of Maltose

As indicated in Table 19**,** 250 mM maltose gave the highest embryonic calli (somatic embryos) formation. There was also a considerable variation in the response of the explant sizes to the various maltose levels tested. In this experiment the highest (100 %) somatic embryos formation was obtained from intermediate size, using 250 mM maltose. This implies that the requirement of the various explants for maltose level depends on their initial size. For instance, 150 mM maltose was suitable for S (97.2, 77.8 %) and 350 mM for L sizes (97.4, 76.9 %). However, the regenerating calli at the highest maltose level were abnormal, producing curled leaves. Therefore, 250 mM was taken as an optimum for our continuing experiments.

Table 19. Effect of maltose levels on somatic embryo formation and plant regeneration for variety DZ-01-196.

Embryo size	150 mM				250 mM				350 mM			
	EM NO.	CF (%)	SE (%)	PR (%)	EM NO.	CF (%)	SE (%)	PR (%)	EM NO.	CF (%)	SE (%)	PR (%)
Small	30	100	97.2	77.8	38	100	81.2	60. 0	30	100	96.7	52.6
Interme.	30	100	85.2	66.7	39	100	100	100	30	100	68.9	62.1
Large	30	100	53.8	42.3	45	100	84.4	75.6	30	100	97.4	76.9

Em - zygotic embryo, CF - callus formation per zygotic embryos cultured, SE - somatic embryo formation per zygotic embryos cultured, PR, plant regeneration, Interme- intermediate

Experiment 3. Effect of 2, 4-D

High variation in embryonic calli formation and regeneration at the various levels of 2, 4-D tested were observed (Fig. 11). 10 µM 2, 4-D was suitable for fast (14 d) and high efficiency (80-98.6%) embryogenesis for both varieties. The highest response (98.6%) of somatic embryogenesis. was obtained from the intermediate size embryos of variety DZ-01-196 followed by Fesho with 80 % at the same level of 2, 4-D. The type of callus at 20 µM 2, 4-D was distinctly varied in morphology by inducing large yellowish aggregates of calli, which only rarely and lately formed somatic embryos and regenerated slowly. Therefore, 10 µM 2, 4-D was taken as an optimum for further experimentation.

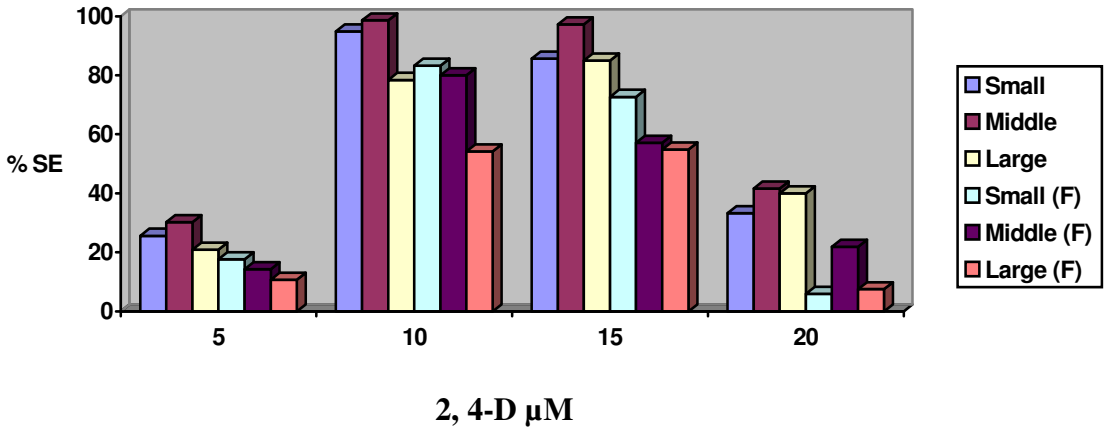

Fig. 11 Effect of 2, 4-D on somatic embryo (SE) formation from different zygotic embryo sizes of varieties, DZ-01-196 and Fesho (F).

Experiment 4. Effect of Phytagel

The type of embryogenesis (direct or indirect) and duration of somatic embryos formation varied among the sizes of embryos based on each phytagel level tested. For instance, at 0.2 %, intermediate and large sized embryos showed slow growth (4 weeks) but larger sized calli, formed somatic embryos in indirect mode of embryogenesis. However, in 0.6 % phytagel level, callus induction was slower (more than 5 weeks) but embryogenesis was direct for all sizes. The percentage of somatic embryo formation (Fig. 12a) and regeneration (Fig. 12b) highly varied among the treatments, especially for shoot formation. The highest somatic embryo formation and shoot or plant regeneration (100 %, 98.2 %) was obtained from treatment 0.5 % phytagel of intermediate sizes (Figs. 12a and 12b).

Although each size showed its own preference of phytagel concentration for high somatic embryo formation (Table 20 and Fig 12a) and plant regeneration and (Table 20 and Fig 12b), generally the intermediate sizes out-performed by showing 98.2 % regeneration at 0.5 % phaytagel concentration followed by large size (78.4 %) at 0.4 % and S (57.6 %) at 0.5 %. Among the tested levels, the regeneration capacity of calli at 0.5 % was higher than the rest. Therefore, it is necessary to measure the concentration of phytagel according to the size of the explant used.

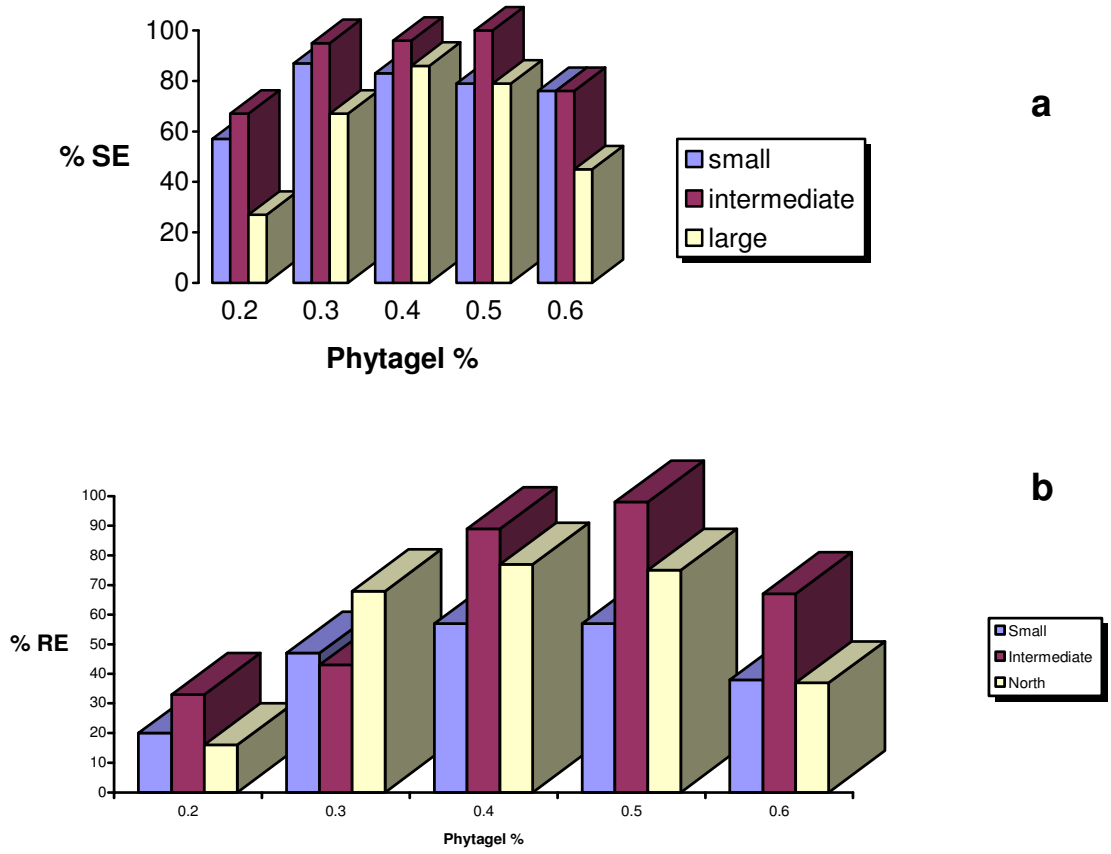

Fig. 12 Effect of phytagel on somatic embryo formation (**a**) and plant regeneration (**b**) from the three embryo sizes of variety, DZ-01-196.

Table 20. Percent of callus induction, somatic embryo formation and shoot regeneration among the three sizes of embryo explant of variety DZ-01-196 at various levels of phytagel.

Phy %	0.2				0.3				0.4				0.5				0.6			
Em size	EM No	CI %	SE %	R %	EM No	CI %	SE %		EM No	CI %	SE %	RI %	EM No	CI %	SE %	R %	EM No	CI %	SE %	F %
S	44	97.9	56.8	2	72	87.5	87.5	4	70	92.9	82.9	5	92	100	79.3	6	68	88.2	76.4	3
I	49	100	67.4	3	49	95.0	95.0	4	73	97.3	95.9	8	57	100	100	9	78	82.1	75.6	6
L	48	100	27.1	1	57	87.7	67.0	6	63	95.2	86.2		76	96.1	78.9		75	84.0	45.3	
									6.3				6.30				6.30			

Phy - phytagel, Em - zygotic embryo, CF - callus formation per zygotic embryos cultured, SE - somatic embryo formation per zygotic embryos cultured, PR, plant regeneration, S –small, I- intermediate, L - large

Experiment 5. Effect of Media

The two media salts highly varied in the efficiency of regeneration of the three varieties. K99 outperform MS medium salts for somatic embryogenesis in all the varieties tested. The number of regenerated calli in MSSM (MS shoot regeneration medium) was better than K4NB (Table 4) for varieties Fesho and DZ-CR-37**.** A similar pattern for MS medium was noted in the responses of embryo sizes. The components of K99 were used since, regeneration was highly reduced in MS medium compared to K99 (Fig. 13).

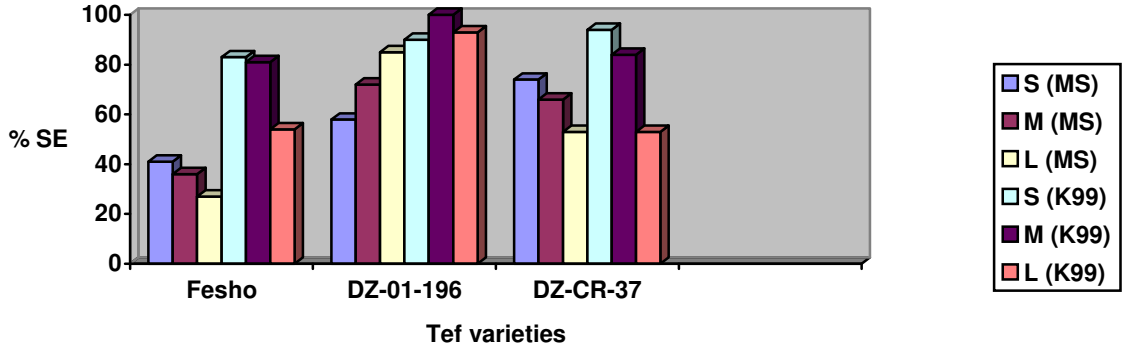

Fig. 13 Comparison of MS and K99 minerals salts on percent of somatic embryo formation using the three sizes of embryo of varieties, Fesho, DZ-01-196 and DZ-CR-37.

4.2.3 Regeneration and number of plantlet produced per explant:

The calli which were not embryogenic had no capacity to regenerate. Therefore, only embryonic calli with matures SE (which able to convert to shoots) were transferred to K4NB regeneration medium and MSSI. These embryos have a normal morphology and accumulated enough storage materials and acquired desiccation tolerance at the end of maturation to develop into normal plants. Only the somatic embryos were developed into small plants comparable to seedlings on culture medium lacking plant growth regulator. However, there are cases where auxin and cytokinin stimulate germination. In this case BAP was the essential growth hormone for the regeneration when extra glutamine is added. Shoots and roots were regenerated within 5-7 days (Fig. 14a). The capacity of somatic embryos to regenerate shoot and root and the high number of

plantlet produced per explant was highly depended on the genotypes, culture media composition and the size of embryo.

Generally, the intermediate size embryos in the modified K99 medium gave the highest number of shoots (Fig. 14b). The highest number of regeneration of up to 565 with a mean (141) plantlet per explant were obtained from the single intermediate size somatic embryos of DZ-01-196 (Fig. 14c) after 8 weeks of sub culture in RM with subculturing at two weeks interval. The reason for big difference between 565 and 141 is due to the further sectioning of single regenerating calli (Fig. 14b) into several pieces and culturing them in separate petri dishes in the case of the 565 plantlet per explant.

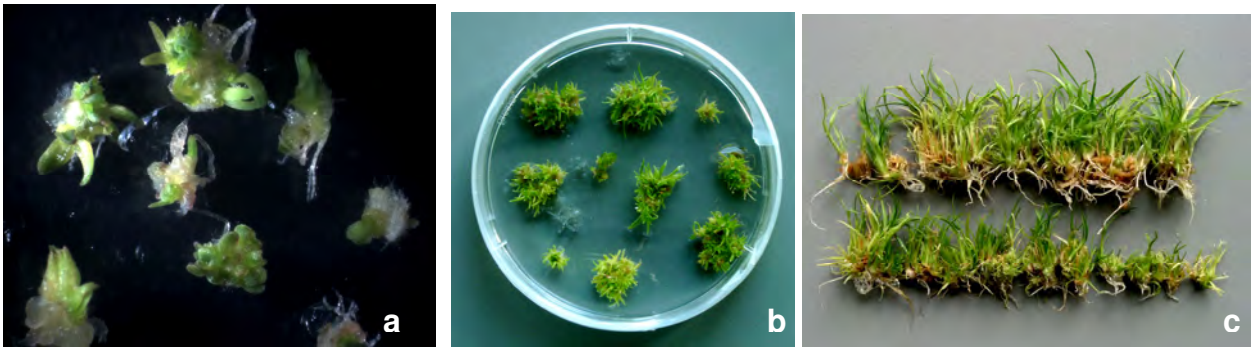

Fig. 14 Regeneration of somatic embryos **a)** regeneration of intermediate size after 5 days on RM medium **b)** four weeks later **c)** plantlets derived from single explant after sub culture for 8 weeks on fresh medium.

Sub culturing was essential to increase productivity of secondary somatic embryos. Normally the small and intermediate sizes embryonic calli that were transferred to RM medium (light) started shooting and rooting after 5 days. When this embryonic calli were transferred to light and further increase in size and proliferation of new secondary somatic embryos that subsequently increased the number of regenerants per explant were observed. Embryonic calli, which were transferred to MS shoot induction medium however, showed low (0-25 %) regeneration rate and did not show secondary somatic embryo development. Besides, low efficiency of plant regeneration, (2-10 plantlets per explant) was obtained in this regeneration medium.

There was also considerable variation in the percent of regeneration of each variety. For instance, Fesho's embryonic calli produced pigments within two weeks in K4NB RM medium, and regenerated to only stunted shoots, producing zero to five plantlets per callus. This was improved by 80 % when the calli were first transferred to either of the media supplemented with only 0.1 µM 2,4-D for four weeks and then transferred to media supplemented with 1 mg/l GA_3 for two weeks and again transferred to MS medium containing 10 % of BAP and (0.1 µM) 2,4-D. With this system plantlets regenerated from Fesho increased to an average of 30 and a maximum of 91 vigorous plantlets per explant. The latter medium improved the regeneration capacity of old (77d), browny and only rooted calli of Fesho to freshly formed whitish secondary somatic embryos and green shoots within two weeks. For variety DZ-CR-37 up to three-fold of increase in the number of plantlets per callus (120) was observed when the embryos were cultured in K99.

The majority of matured somatic embryos (Fig. 15a), which were isolated and placed in the germination medium resulted in normal germination of shoots and roots simultaneously (Fig.15b).

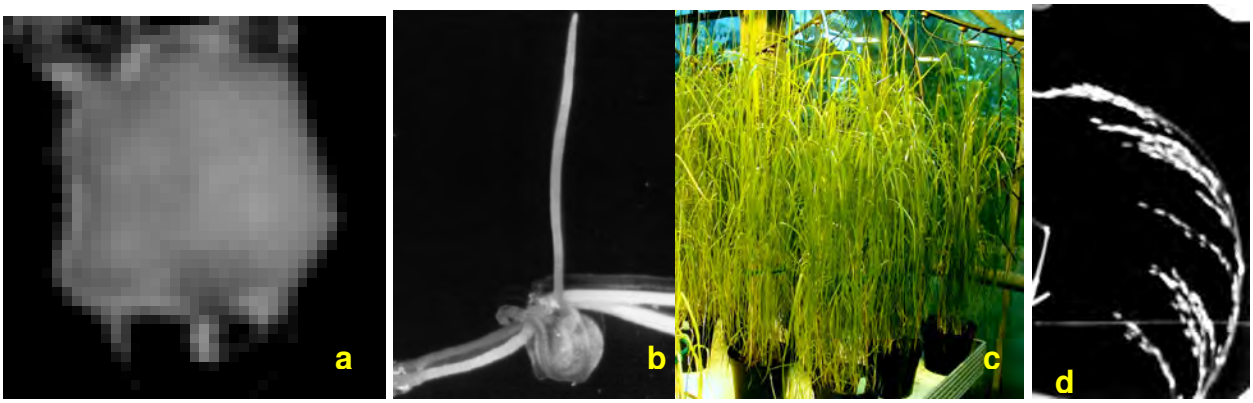

Fig. 15 Germination of somatic embryos **a)** matured isolated somatic embryos resembling natural embryos of tef **b)** simultaneous germination of the isolated somatic embryos to shoot and roots **c)** normally grown tef plants from immature embryo culture of tef **d)** fertile panicle of tef (DZ-01-196) obtained from embryo culture.

All transplanted plants derived from each experiment grew to morphologically uniform (Fig. 15c) and gave normal fertile (Fig. 15d) plants.

4.3 Discussion

This is the first report for high and efficient plant regeneration from immature embryos of tef. In this protocol, tef immature embryos exhibited high totipotency due to the suitable modified K99 media combination developed. This is due to the use of the appropriate concentration of 2,4-D, 10 μM; phytagel, 0.5 %; maltose, 250 mM; glutamine, 6 mM and choosing appropriate sized explants for high efficiency somatic embryogenesis and plant regeneration.

In the present study, somatic embryogenesis was initiated only when scutellum was placed up side. As Williams and Maheshwaran (1986) stated for other plants, somatic embryos of tef differentiated directly either from the explant without an intervening callus phase or indirectly after a callus phase. According to these authors, explants from which direct embryogenesis is most likely to occur from immature explants include microspore (microsporogenesis), ovules, zygotic embryos and seedlings.

4.3.1 Embryo size: Developmental stage of the explants is reported to be a crucial factor for *in vitro* regeneration in many cereal crops (Lu et al., 1984, Chang et al., 2003). Chang et al., (2003) reported the effect of size of immature barley embryos to affect the efficiency of plant regeneration. They showed that the smaller embryos (0.5-1.0 mm) had the highest regeneration potential compared to larger sizes (1.6-2 mm).

In addition they stated that the smaller embryos not only solved the low regeneration efficiency but also the albinism problem. Normally the sizes of somatic embryonic calli (SE) of tef were according to the initial explant sizes and the level of the media composition, mainly of the glutamine, 2, 4-D and phytagel level used. However, in general immature embryos are fast and highly responsive to somatic embryogenesis (10-14 days) resulting in high efficiency plant regeneration. This could be due to the highly embryonic nature of the immature embryos. The larger sized tef embryos are composed of proembryonic masses, also latter developed through callus phase to somatic embryos. In the attempt of tef transformation, immature embryos can be used as a suitable explant since they exhibited high regeneration within a short period than matured explants.

4.3.2 Growth hormone, 2, 4-D: Embryogenic cells have been formed in only auxin containing media and explants continued proliferation forming proembryonic masses within few days after culture. Embryogenesis was not observed in auxin (2, 4-D) free medium. Similarly, earlier reports included young leaf (Mekbib et. al., 1997) and young leaf and root segment cultures (Bekele et. al., (1995). One mechanism whereby auxin may regulate embryogenesis is through acidification of cytoplasm and or cell-wall (Kutschera, 1994). However, as auxin is important in the induction of embryogenesis, it is inhibitory for the development of proembryonic masses into somatic embryos (De-Vries et al., 1988). Despite of this, it should be noted that auxin is rapidly taken up from the medium so that depletion of auxin in the culture medium starts already after few days (Von et al., 2002) thus, somatic embryos are formed in the absence or scarce level of this hormone. This is in agreement with De-Vries et al., (1988) that, if cultures are not transferred to fresh medium each week, the development of somatic embryos may start. But this was not always true with what was observed in the case of variety, Fesho. This genotype required a subculture to fresh new induction medium of the same composition after two weeks of culture initiation in order to initiate somatic embryos.

The highest SE formation rates (78-100 %) for varieties DZ-01-196 and DZ-CR-37 were achieved when 10 or 15 μM 2, 4-D were used. The increasing of 2, 4-D to a higher level (20 μM) reduced the somatic embryos formation of the immature embryos of tef. Fesho's subculturing requirement together with the supplementation of (0.1 μM) 2, 4-D in the regeneration medium highly improved embryonic potential and regeneration. This indicates that the response of tef genotypes for a given medium or culture protocol is different from each other.

4.3.3 Glutamine: Addition of amino acids to culture media has been reported to improve plant regeneration. Glutamine, asparagine and proline are the most frequently used amino acids (Hagio, 2002). Besides, glutamine is one of the common organic nitrogen sources (Franklin and Dixon, (1994) that is used in cereals. In this study, results illustrated the level of glutamine and phytagel as a limiting factor for somatic embryo formation and regeneration in tef embryo

culture. Exogenous glutamine has previously shown to benefit the growth of asparagine callus (Hunault, 1981). Similarly, the high level (6 mM) of exogenous glutamine greatly stimulated the production of somatic embryo aggregates and maintained the embryonic tissue potential, may be through an increase in endogenous glutamine levels. These may indicate that, embryonic cells might have a high metabolic requirement for glutamine for synthesis of other metabolites and proteins (Ogita et. al., 2001). According to these authors, high level of exogenous glutamine increased the synthesis of certain macromolecules or metabolites that are essential to the maintenance of embryonic properties of *Cryptomeria japonica*. Ogita et al., (2001) stated that high glutamine (2400 mg/l) containing medium increased the dry weight and embryogenus level, thus generating the embryogenic aggregates of japonica rice cells. High concentration of glutamine in the analysed embryogenic cell lines and cultured tissues of carrot (Kumarda and Harada, 1984; Joy et al., 1997), white spruce (Joy et al., 1997) was also reported. Thus according to the experiment carried out by the former reporters a higher concentration of glutamine enhanced proliferation of the embryogenic tissue and might have increased the synthesis of certain macromolecules of metabolites that are essential to the maintenance of embryogenic properties of cells. The higher embryogenesis in tef might also be due to similar reasons.

4.3.4 Effect of media: Various media combinations were reported to be suitable for immature embryogenesis in many crops. These include MS (Murashige and Skooge, 1962) N6 (Chu, 1978) and B5 (Gamborge et al., 1968). K99 which is the recently developed basal medium by Kumlehn (unpublished data) found to be very efficient for somatic embryo formation for the immature embryo culture of tef cultivars than the most known ones. According to the results obtained from this study, variation of somatic embryo formation and regeneration plants among the genotypes were greater in regeneration medium than in the induction medium. As described in the result section, Fesho's somatic embryos were unable to regenerate when directly transferred to K4NB regeneration medium. Therefore, suitable induction and regeneration medium combination are important factors in somatic embryo initiation and regeneration of tef cultivars. Genotypic variation in regeneration was also reported in two rice cultivars. Japonica rice showed higher rate of shoot regeneration compared to Tongil type in that regeneration in MS basal medium was superior to modified N6 basal medium under 2 mg /l kinetine and 5 mgL-1 NAA in rice scutella derived callus culture (Cho et al, 2004).

All the transplanted plantlets in the greenhouse were uniform, normal and fully fertile. There was no obvious morphological based somaclonal variation observed from this experiment. Further separation of the embryonic calli in to small pieces and subsequent culture in fresh media increased the number of plantlets per callus. Single mature somatic embryos were also dissected from the embryonic callus in order to investigate their normal germination on germination media without growth hormones. Tef immature embryos with K99 medium can be used as a protocol for immature embryo embryogenesis and plant regeneration especially during transformation studies where high efficiency of regeneration is needed.

In this protocol the tissue culture system of tef immature embryo culture has been significantly improved.

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Chapter 5

Androgenesis in tef

Part I: Anther Culture

5.1 Materials and Methods

5.1.1 Genotypes: Nine tef varieties five of which were landraces namely; Keymuri, Bunniye, Dabi, Alba and Fesho and four improved varieties, DZ-01-196, DZ-01-354, DZ-01-974 and DZ-CR-37 as well as the wild relative, *E.mexicana* were the study materials used in this study. The landraces were chosen for their colour markers of anther, lemma and culm (Table 21). The wild relative, *E.mexicana* was included since it matures very early, showing vigorous growth (than most other wild species) and produces high number of florets per panicle.

Table 21. Variations in anther, lemma colour and days to maturity of the study materials

Genotypes	Lemma colour at flowering	Anther colour at anthesis	Maturity days	Source
Keymuri	dark red to very deep red	very dark red	90-105	Ebba, 1975
Bunninye	variegated, pale yellow-green with dark reddish purple suffusion	deep purple to dark-reddish purple	75-85	Ebba, 1975
Dabbi	variegated, greyish olive green with dark purple tips and margins	light reddish purple	80-95	Ebba, 1975
Alba	pale yellow green	dark greenish yellow	95-120	Ebba, 1975
Fesho	dark purple to deep purple	blackish purple	75.85	Ebba, 1975
DZ-01-196	variegated, yellowish white with red tips and margins	deep red	80-113	Tefera et al., 1995
DZ-01-354	yellowish white	yellow	85-130	Tefera et al., 1995
DZ-01-974	yellowish white	yellow	76-138	Tefera et al., 1995
DZ-CR-37	yellowish white	yellow	82-90	Tefera et al., 1995
E. mexicana	yellowish white	deep red	65-75	Personal observation

5.1.1.1 Growth condition: Plants were grown in the green house of the Institute of Biozentrum Klein Flottbek und Botanischer Garten (AMPII), Hamburg, Germany. The growth condition of the plant material was as described in Chapter 3. However, application of fungicides and pesticides was reduced in this study to avoid damage of microspores.

5.1.1.2 Identification of the appropriate harvesting stage of panicle: A critical step in androgenic culture is the selection of appropriate spike, tassel or panicle at the appropriate harvesting stages. The appropriate stage where the maximum number of explants (anthers or microspores can be isolated is essential and for this reason the following five stages were investigated.

Stage 0, booting stage, before panicles emerged

Stage 1, 1-5 cm panicles emerged out of the sheath

Stage 2, 5-10 cm, where panicles emerged 1/3 out of the sheath

Stage 3, 10-14 cm, where panicles emerged 1/2 out of the sheath

Stage 4, 15-20 cm, where panicles emerged 2/3 out of the sheath

5.1.1.3 Surface sterilization: Healthy and vigorous panicles from the first tillers were harvested from the greenhouse grown plants and were either wrapped in aluminium foil and stored at 4°C for cold pre-treatment or surface sterilized as described in chapter 3.

5.1.1.4 Anther growth stage determination: Variety DZ-01-196, which has pigmented anthers, was selected as starting material to distinguish the stages of anther development. For instance, the matured anthers of this variety have deep-red colour while, the very young ones have shiny light green anthers. The consecutive anthers development ranged between these two colours. Anthers were collected from various stages of panicle with florets at various growth stages in each spikelet. Classification of anther development from the respective stage of panicle was done at early anthesis stage. These were categorized and numbered, for instance, samples taken from panicle stage 3 were divided further into three parts (bottom, intermediate and top). Flower number one is given to the basal floret of each spikelets and the next is numbered as flower number 2 and continued in ascending order until last flower.

5.1.1.5 Microspore growth stage determination: The three anthers found in each floret were immediately placed on a slide containing a drop of 0.3 M mannitol and 25 µM $CaCl_2$ solution. After cover slip is kept carefully on the slide a slight squash is made to disperse the pollen grains in the anther. The microspores may then quickly be observed under the stereo microscope at 40 X magnification. To distinguish the similarity between tef and *E. mexicana* pollens, freshly shed pollens from matured anthers were examined.

5.1.1.6 Anthers isolation and culture condition: Anthers were isolated from each developmental stages of panicle with the exception of booting stage. Spikelets were placed under dissecting microscope and the three anthers were isolated from each flower. The basal portion of the florets was cut using fine blades and all sex organs (pistil and stamen together) were slightly pushed towards the cut. These were cultured in two types of culture condition namely isolated anther culture and co-culture with ovaries according to their stage of growth. Thirty to forty florets, equivalent to 90-120 anthers without any pistils and same but with pistils were cultured using liquid or solid medium.

5.1.2 Pre-treatment for anther culture: From the preliminary observation, it was known that no better result was gained from the hot pre-treatment (32°C for 24 hrs) compared to the cold (4°C for 1-4 weeks). Therefore, only the latter was used to optimize the appropriate durations. The following 4 treatments were conducted.

Pre-treatment 1. Control, no pre-treatment, fresh anthers were directly cultured at 25°C in the dark.

Pre- treatment 2. Panicles after harvest were immediately wrapped in aluminium foil and stored at 4°C for 1-4 weeks or alternatively panicles after surface sterilization were cut into 5 cm segment and placed in 44 cm petri dishes with moist filter paper, sealed and kept in cold (4°C) condition for 1-4 weeks in the dark.

Pre-treatment 3. Anthers from pre-treatment 2 were excised and cultured on the induction media and stored in cold (4°C) condition for one week before being transferred to 25°C under dark.

Pre-treatment 4. As experiment 3 but, stored for two weeks before being transferring to normal culture condition, 25°C dark.

5.1.3 Co-culture: Tef ovaries with similar growth stage to anthers were co-cultured with the anthers in order to observe the effect of co-culture and feeding. Pre-conditioned media (Zheng et. al., 2001) was also used with various ovary numbers (2, 4, 6, 10 and 40 per well).

5.1.4 Media: Three basal media, N6 (Chu, 1978), MS (Murashige and Skoog, 1962) and K99 (see Table 4) with and without 2, 4-D in both state of media (liquid and solid) were initially compared. Several experiments were carried out latter using only K99 (Kumlehn unpublished) containing 9.2 µM 2, 4-D.

5.1.5 Data Collection: Anthers development was observed in a week interval. Anthers, which dehisced microspores in culture and the division of these microspores to 4-8 cells stage, were considered as responsive explants. The development of microspores and the induction of micro calli were counted per cultured anther after three to four weeks. Photos were taken using light microscope.

5.2 Results:

5.2.1 Growth condition of the donor parent and panicle harvesting stage: Healthy, vigorous and freshly harvested panicles from the first emerged flowers of the panicle at stage 3 (1/3 emerged out of the boot (Table 22, Fig. 16) were the best sources of anther for androgenesis in tef. Table 22 shows the stage of anther development at various stages of panicle emergence. Since the number of spikelets per panicle is higher in late maturing varieties, high number of explants at the right stage can be collected from the late varieties than the early ones. However, repeated collection can also be made form the early genotypes for repeated experiments

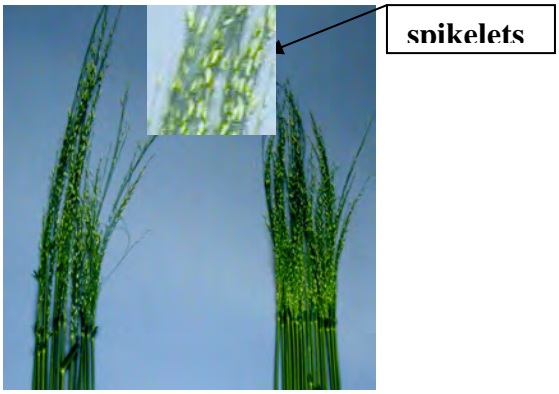


Fig. 16 Appropriate stage of panicle growth. of DZ-01-196 (left) and DZ-CR-37 (right)

Stage of Panicle	Stage of panicle (emergence out of the sheath)	Status of floret at each panicle parts		
		Top	Intermediate	Bottom
0	booting (not emerged)	few flowers available	no flower available	no flower available
1	1-5 cm emerged	many flowers available	few flowers available	no flower available
2	5-10 cm, 1/3 emerged	only above the 5th flower is at right stage	only above the 3rd flower is at right stage	immature flowers
3	10-14 cm, 1/2 emerged	majority are at right stage	majority are at right stage	some are at right stage
4	15-20 cm, 2/3 emerged	majority are pollinated florets	few are at right stage	majority are at right stage

Table 22. Stage of growth of tef florets at different panicle growth stages & panicle parts.

5.2.2 Anther size and number of pollen grains per anther: Tef florets are hermaphrodite with three stamens surrounding the single pistil (Fig. 17a). The anthers have two lobs and divide in to two (Fig. 17b). The size of the normal matured tef anthers varies from 3-5 μm and the average size of matured normal pollen is approximately 0.11 μm. The number of pollen grains in the tef anthers is very small compared to other cereals. In normal and fertile anther of tef a maximum of 100 and an average of 50-75 pollen grains are found (Fig. 17c).

5.2.3 Classification of tef anthers and microspores according to their growth stages: Table 23 shows the developmental stage of florets and anthers at early anthesis of the intermediate spikelets. As can be seen from this Table, anthers can be cultured from flower number 3-4 to study the effect of different culture conditions.

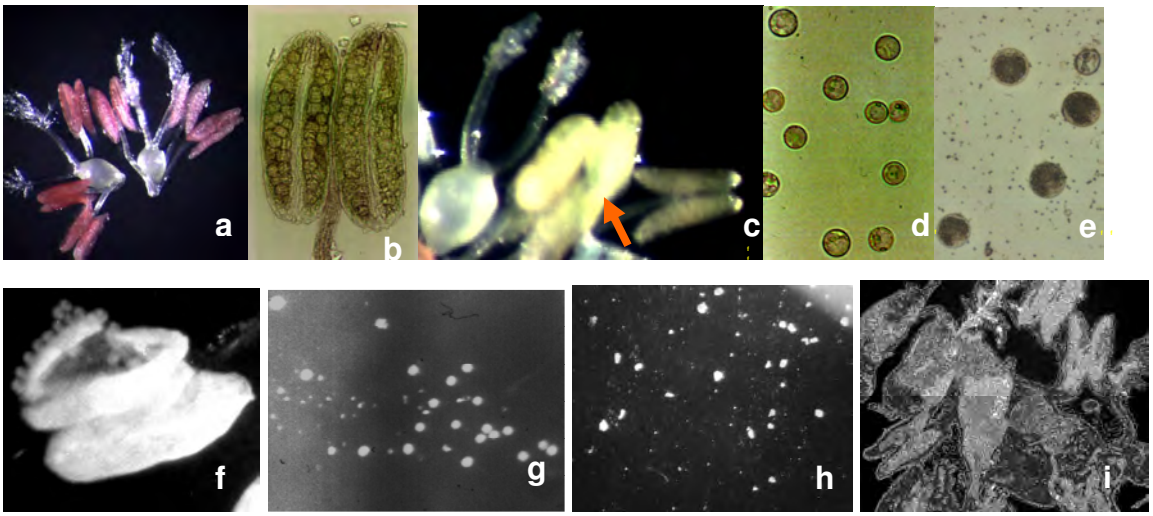


Fig. 17 Development of microspores in anther culture of tef **a)** hermaphrodite florets of tef **b)** matured anther of tef containing pollen grains **c)** immature anther at uni nucleate stage (arrow) **d)** viable microspores at right stage (uninucleate stage) **e)** matured pollen grains filled with starch grain at trinucleate stage **f)** dehiscence of anther *in vitro*, showing the release of microspores at globular stage **g)** globular microspores growing in liquid culture **h**) microspores growing to micro calli **i)** collapsing of the enlarged co-cultured ovaries.

Table 23. Microspores developmental stage of the various florets on a spikelet. Samples were taken from the intermediate part of stage 2 (1/3) panicle that emerged out of the sheath).

Floret N	**Microspores developmental stage at various florets.**
0	already self pollinated floret and at least 2 of the 3 anthers shed their microspores
1	floret at 1-2 days before anthesis, containing big, coloured and matured anthers (microspores at trinucleate stage)
2	floret at 3 days before anthesis and anthers as large as No 1. but, deep green coloured (majority of microspores are at bi-nucleate stage with few late uninucleate stage)
3	florets at 4-5 days before anthesis, anthers relatively smaller size than No.2 and light gr colour (early -late uni-nucleate stage microspores (Fig 17d)
4	young florets containing anthers relatively smaller than No.3, and light greeni

No.2containing various

stages, early uninucleate, empty microspores, S size microspores to early released tetrad st

5 very small florets with very small shiny anthers at megasporosite stage These florets a young

and developed no distinct sex organs.

NB: Floret maturity starts from the base of the spikelet, i.e. from flower number 0

5.2.4 *In vitro* development of the cultured anthers: Anther dehiscence and the release of pollen grains in a culture depend on the stage of the initial explant and pre-treatment used. All large sized (matured, trinucleate stage) anthers burst and released their pollen grains after two to three days of culture. These stages of anther are too old for androgenesis because, microspores already undergone meiosis II to form vegetative and generative cells and are filled with starch grains (Fig. 17e). The intermediate and small sized anthers treated in cold were burst and shed their viable microspores at uni-binucleate stages after one to two weeks of dark culture respectively. The shed microspores were floated in the liquid K99 medium or sticked to the edge of the anther on the surface of the solid medium (Fig. 17f). Of these, 10-15 percent of the shed microspores grew to globular stages (Fig. 17g) after one to two weeks. Thus, it is considered that these young stages are suitable for anther culture. Small sized anthers, often failed to burst and microspores start division while they are inside the anther lobs. In general, the growth and division of the microspores mainly depended on the type and duration of the pre-treatment tested and the media used.

5.2.5 Pre-treatment: None of the microspores from the freshly cultured (untreated) and four weeks cold pre-treated anthers and panicles showed division (Table 24). The highest division was observed from the two weeks panicle treatment in cold followed by one week pre-treated anthers in both varieties (DZ-01-354 and DZ-01-196). Variety DZ-CR-37 was also included in this experiment but was not responsive to the pre-treatment and the majority of its anthers did not show any development for more than 4 weeks and later turned to brown and died. The long pre-treated panicle segments in the petri dishes or in the aluminium foil usually dried or infested by fungus. Therefore, this pre-treatment method was omitted after the initial stage of the study.

5.2.6 Media: Anthers were shrinked and turned brown faster in N6 medium (Chu, 1978) compared to MS (Murashige and Skoog, 1962) and K99 (Kumlehn unpublished) media. The dividing microspores induced micro calli (Fig. 17h) in both liquid and solid media of MS and K99 only when 0.1 µM 2, 4-D was added. There was no response at all in media containing no growth hormones and media containing only BAP. Both late maturing genotypes (DZ-01-974 and DZ-01-354) showed sign of development from the one week pre-treated anthers which kept in cold. However, the transfer of these micro calli to solid or to fresh liquid media did not help in the induction of bigger calli or direct embryony.

Table 24. Effect of pre-treatment on percent of callus induction of the anthers co-cultured with ovaries. Varieties DZ-01-354 and DZ-01-196 on K99 medium.

Pretreatm 4°C	**Number of explant cultured, microspores division and % of calli induced**										**Remark**
	DZ-01-354					**DZ-01-196**					
	No Ant	**No. of ovarie**	**No. of ms**	**No of ms divisio**	**% o CI**	**No. of Anthe**	**No. of ovarie**	**No. ms**	**No.of ms divide**	**% of CI**	
Fresh	**500**	**100**	**690**	**0**	**0**	**500**	**100**	**500**	**0**	**0**	**Co-cultured o ovaries enlarge**
1 week	**146**	**450**	**1597**	**146**	**2.9**	**315**	**114**	**320**	**10**	**7.8**	**Ovaries enlarg**
2 weeks	**495**	**195**	**640**	**19**	**9.2**	**495**	**195**	**243**	**25**	**4.1**	**Ovaries enlarg**
3 weeks	**485**	**160**	**150**	**1**	**0.6**	**471**	**132**	**98**	**1**	**1.0**	**Panicle usually dried, anthers turned yellow and ovaries do not enlarge**

NB: CI= callus induction, ms = microspore

5.2.7 Effect of co-culture: There was no variation in the number of responding anthers in both isolated and co-culture conditions**.** The effect of co-culture was not clearly observed as it does for other cereals such as wheat (Zheng et. al., 2001). Pre-conditioned media (Zheng et. al., 2001)

using various numbers (2, 4, 6, 10 and 40) of pistils of tef per well, also did not affect the growth of the cultured anthers and thus no additional advantage was observed by co culturing. Rather, the co-cultured pistil enlarged and swells approximately to the size of a tef seed and finally collapsed in culture on the 5th day (Fig. 17i).

5.2.8 Genotypic response: At the beginning of the preliminary study, the fresh and 1-4 weeks cold pre-treated isolated anthers were cultured in various types of solid media and at various culture conditions. However, five of the ten genotypes tested (Dabbi, Bunninye, Key muri, DZ-CR-37 and *E. mexicana*) showed no growth or release of microspores within four weeks. These could be due to the growth condition of the donor plant which was 16 h light and 70 % relative humidity. The normal growth condition of tef is a maximum of 12h light and 50-55 % relative humidity at the day time. Under the present green house condition, Fesho and *E .mexicana* were the only genotypes that grew normal and repeated .experiments were done using these early maturing genotypes but no response was observed from the 500 cultured anthers of each. Only few microspores of the late varieties (DZ-01-974, DZ-01-354**,** DZ-01-196, Alba and *E.mexicana*) showed division and micro calli development. So the experiment was interrupted and only isolated microspore culture was continued.

Part II: Microspore culture

5.3 Materials and Methods

5.3.1 Plant growth: Donor plants growth was as described in tef anther culture experiment (see part I of this chapter). Panicles were harvested from the vigorous main tillers at three stages, namely 1, 2 and 3.

5.3.1.1 Genotypes and surface sterilization: Based on the response of anther culture and maturity only five genotypes, DZ-01-196, DZ-CR-37, DZ-01-974, Alba and Fesho were chosen for this study. Sterilization was as presented earlier for anther culture.

5.3.2 Microspore isolation using waring micro blender: Microspore isolation from tef panicles using mortar and pestle was not possible as it does for barley spikes. Therefore, micro

blending technique using a Waring Micro Blender, (Fig. 18a arrow) (Eberbach Corporation, Ann Arbor, Michigan, USA), and a modified protocol of Mordhorst and Lörz (1993) and Jähne and Lörz (1995) were used. The number of microspores was increased after some modification during the time of blending and panicle preparation. Initially up to 10 pre-treated panicles were cut into 1 cm segments and transferred to a cold pre stored blender and 10 ml of cold 0.4 M mannitol solution was added and microspores were released by blending three times for 5 sec at a low speed. Microspores suspension was filtered through 50 µm sieve and the debris were re-blended by adding 10 ml mannitol solution. The suspension was centrifuged for 10 min at 10000 rpm speed and the supernatant was removed.

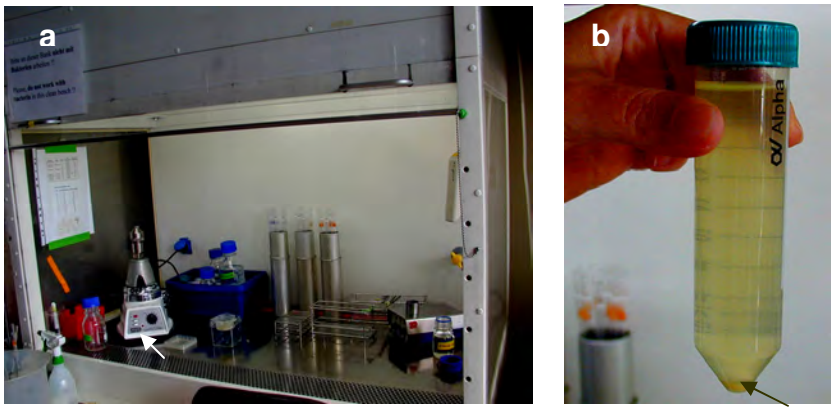


Fig. 18 a) Microspore isolation equipments, under laminar flow, Micro blender (arrow) **b**) microspores pillet (arrow) after centrifugation.

The pellet (Fig. 18b) was resuspended in 3 ml of 0.5 M maltose and the solution was transferred to 10-mL tube. 1 ml layer of the mannitol was placed slowly on top of the suspension and centrifuged at a low speed (100 rpm) for 15 min. The fraction of viable microspores is located at the mannitol/maltose interphase. The band can hardly be seen if the collected viable microspores are less than a million. The band was collected slowly using 1 mL pipet and immediately transferred to a 50 ml tube and mixed with the medium and cultured in 4 micro well plates.

5.3.2.1 Microspore purification: Two purification methods were compared in order to purify the viable and right stage microspores from the dead and matured ones, so that the total number of viable microspores which will induce embryonic like structures (ELS) can be increased. During purification, viable microspores were separated from the rest by the gradient

centrifugation on different solution, 3.3 x starvation medium +10 % Percoll (Touraev et al., 1996) and 19 % maltose-H_2O (Mordhorst and Lörz, 1993). The isolated microspores were mixed and aliquots of the same volume were placed on top of the purification solution contained in the tube. The tube was centrifuged at low speed for 15 min, and then repeated washing out of the percoll by 0.4 M mannitol were needed to discard the precipitated percoll from the culture.

5.3.3 Media

5.3.3.1 Starvation media: For comparison, two starvation and two nutrient media were tested. The starvation media: K99 with restricted nutrient supply using 0.4 M melibiose or 0.4 M mannitol and 1 mM $CaCl_2$, 2 mM MES, which were suitable for barley and wheat microspores pre-treatments were used. Microspores were isolated from the panicles stored at 4°C for 1-3 weeks and they were tuned to starvation and were cultured at 4°C for 1-3 days.

5.3.3.2 Nutrient media: Two liquid media i) K99C4B (K99 medium supplemented with 0.4 M BAP and with 7 mM glutamine and ii) K99M2, containing half concentration (3.5 mM) glutamine and with no BAP, were compared. Both media were tested with or without supplementing them with 0.1 µM 2, 4-D.

5.3.4 Pre-treatment: Six pre-treatments were tested and were compared with the control

1) Hot pre-treatment of panicles at 32°C for 24-72 h and pre-cultured in starvation media for 1-3 days at 4°C

2) Cold (4°C) storage of panicle for 2-3 weeks

3) Culture of freshly isolated microspores from non-treated panicles in either of the starvation media for 1-3d at 4°C

4) Culture of isolated microspores from pre-treated panicles (2 weeks at 4°C) and pre-cultured in either of the starvation medium for 1-3d at 4°C

5) Microspores isolated from pre-treatment 4 and directly cultured into the nutrient media and stored at 4°C for 1-3 days.

6) Control.

5.3.5 Culture condition: After purification of microspores 1 ml liquid starvation media was added. Then the microspore aliquots was poured in petri dish (3 cm) and 250 µl microspore aliquots was filled in each 4-well dishes (Nunc, Denmark), sealed and pre-treated according to the treatment condition described in 5.3.2.1. After pre-treatment duration the starvation media were replaced by a suitable nutrient medium and incubated at 25±1°C dark to initiate embryogenesis. In order to keep the humidity constant, sterilized water was added in the middle part of the micro wells.

5.3.6 Co-culture: immature pistils or ovaries of wheat were reported to be suitable for use as a feeder in wheat and barley microspore culture (Zheng et. al., 2001; Hönicka, 2001). Therefore, wheat pistils were collected from the immature spikes at nearly the same stage of anther development or a bit older. Pistils were collected aseptically from the middle part of the spikes and panicles. The pistils were co-cultured immediately with the microspores or stored in the nutrient media at 4°C for later use (2 days to one week). Two to ten pistils were co-cultured per well. The other feeding treatment was the use of sporophytically developing barley microspores suspension as described by Kumlehn et al., (1998). One µl suspension from 5-7d old barley, cv. Igri microspores culture was taken and cultivated on the semi permeable membrane of 12-mm millicell inserts (Millipore, Bedford, Mass, USA) that was placed in the four well dishes. The presumed advantage of this technique is based on the assumption that the tef microspores that are cultured in the 4-well dishes will be stimulated and receive feeding effect from the growing microspores of barley through the semi permeable membrane of the millicell. This method is also believed to provide unknown nutrient from the growing embryos to the fresh ones (Kumlehn et al., 1998). After 2 weeks of incubation in the dark, the millicell was removed and the growing embryonic like structures of tef were transferred to solid K99M2 medium for further growth. The following treatments were compared.

Treatment No.	**Treatment of feeding**

1	No feeder and no ovaries
2	Ovary co-culture
3	Immature embryo calli of tef as feeder
4	Suspension from 5-7d old barley, cv. igri microspores as feeder

5.3.7 Data collection:

All the cultures were examined every other day and any contaminated dishes were removed. Dividing microspores and induced embryonic like structures were counted. Experiments were conducted in completely randomized design (CRD). Each experiment contained only one independent variable and 5 to 10 replications. Microspores contained in each petri dish were considered as one experimental unit. Each experiment was repeated 3 times and Mean ±SD were calculated.

5.4 Results

5.4.1 Stages of microspores isolated from the various panicles stages: Normally mixtures of microspores developmental stages were isolated from a given panicle. For instance, few viable and vacuolated microspores (right stages) were found even from the old anthers of flower No. 0 and 1. This indicates that the development of microspores varied even in a given anther lobs. However, the dominating stage clearly depends on the panicle developmental stages (see part I of this chapter). Generally the intermediate panicles at stages 2 and 3 were appropriate to isolate many viable microspores (Fig. 19a) that are at the right stage for the tested genotypes.

5.4.2 Microspores isolation: The number of microspores isolated from tef panicles using the long isolation procedure of micro blending was inadequate and some times resulted in giving no microspore at all. The total number of microspores collected was the main problem to repeat experiments in various treatment combinations. Therefore, several attempts were made to modify the time and frequency of the micro blending used for barley. Increasing the blending frequency and avoiding of the third centrifugation used for barley increased (10%) the number of microspores collected. This method was required because tef panicles possess plenty panicle branches and rachillas. These materials block the fine blending of tef spikelets and many microspores were blocked in the debris on the filter mesh. Removal of these increased the

number of microspores isolated from few to about 1000-10000 (Fig. 19b). The last modification was the cutting of the spikelets instead of panicles and this was found to be more appropriate although, collecting spikelets from each panicle branches was laborious and time consuming. The additional time of blending accounted to three in five second's intervals was better than the once used for barley microspores isolation. Collectively by using this modification more than a million visible microspores (Fig. 19c) were obtained in the interphase band of mannitol/maltose solution (Fig.19d). The number of microspores was increased by two fold when samples collected from the normally grown plants in Ethiopia (Holetta and Debre Zeit experimental fields) were used.

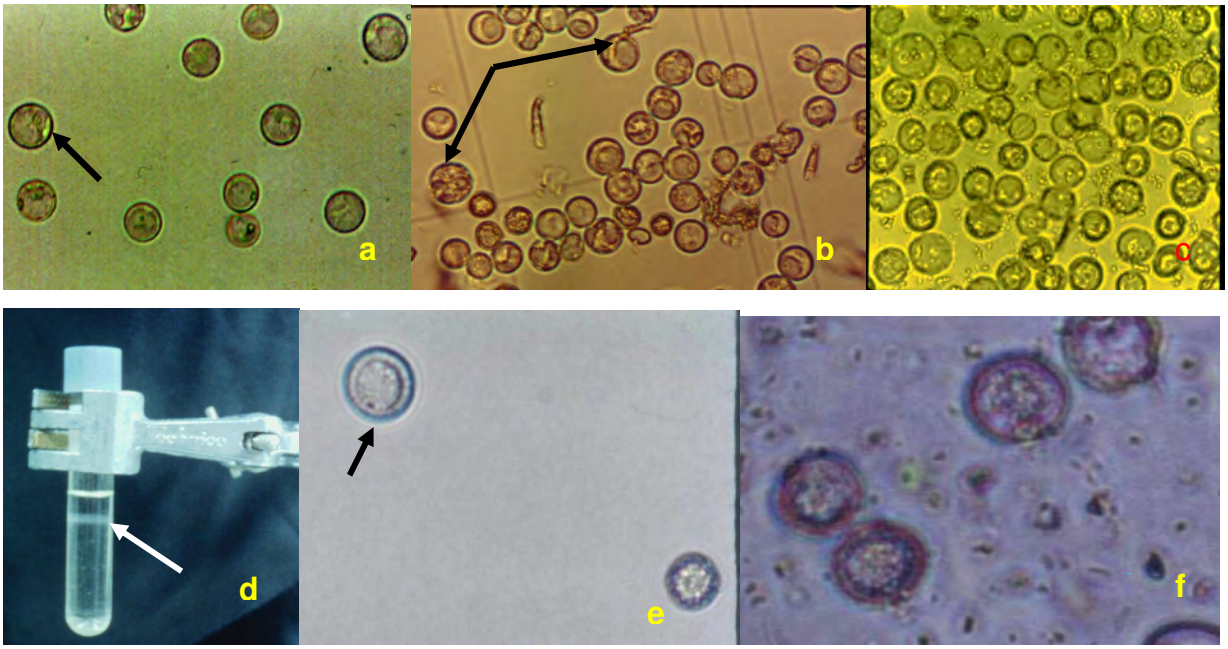


Fig. 19 Microspores isolated using the modified isolation methods and the various developmental stages of microspores **a)** Freshly isolated microspores of Fesho, showing viable microspores with largely vacuolated (arrow) microspores and nucleus at central position **b)** microspores isolated from two weeks pre-treated panicles at 4°C, number of microspores increased after the second modification of the isolation method, some microspores are swollen (arrows) **c)** Number of microspores increased after the third modification of the blending technique **d)** many viable microspores at the interphase band (arrow) **e)** viable microspores identified by its blue exine (arrow) **f)** non viable microspores identified by its reddish or black exine and shrinked cytoplasm.

5.4.2.1 Microspore purification:

The microspores purification solutions allowed separating viable microspores from the dead and old ones. Differences in microspores viability and production of embryo like structures (ELS) after microspores purification were evaluated. Microspores were isolated from 2-3 weeks cold pre-treated panicles. Viability of microspores and production of ELS were very similar and was not affected by the type of purification solution (Table 25). There was no variation in the number of viable microspores isolated from the two purification methods (Table 25). However, the loss of microspores was high when percoll was used because of repeated washing using 0.4M mannitol to remove precipitated percoll from the cultured microspores. The percoll solution is toxic for microspores and most often if not properly washed the majority of the viable microspores die during culture. Moreover, during the repeated washing and sucking, considerable number of viable microspores was lost thus affecting the culture density. Therefore, purification was done by using the maltose method. After purification the growth of the dividing microspores continued until they induced ELS which when transferred to solid media proliferated and grown to bigger calli (Fig. 20h).

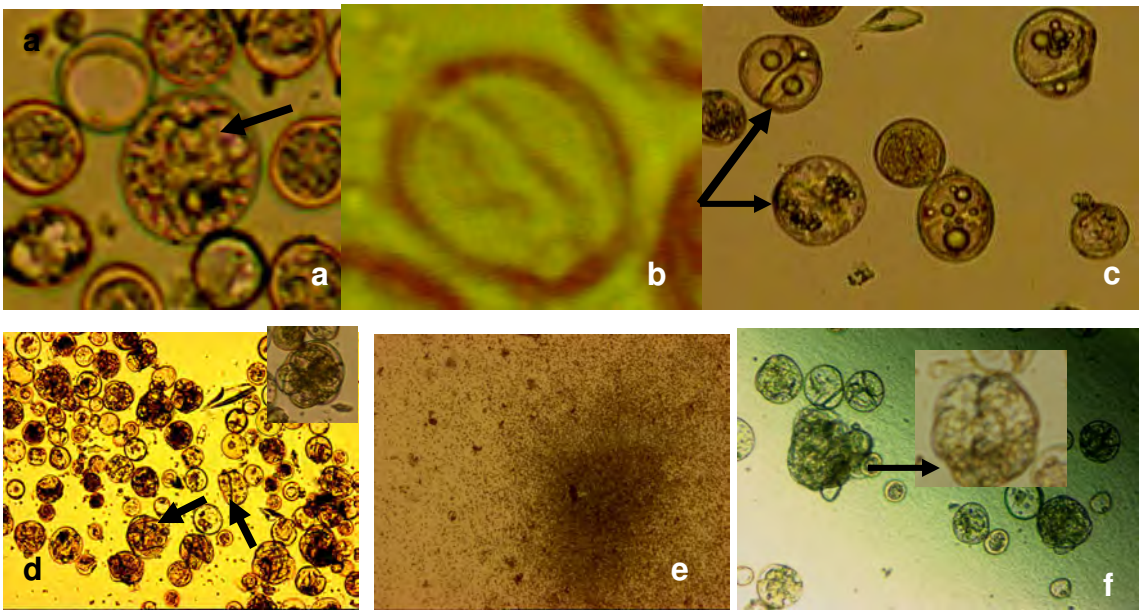
Table 25. Effect of microspores purification solutions on the number of viable microspores and production of ELS.

	Microspores purification solutions	
Parameters	19 % Maltose-H_2O	3.3 x starvation medium + Percoll
Microspores viability	86.31± 4.62	83.81± 6.99
ELS	60.30 ± 16.93	60.70 ± 11.98

Result Mean ± Standard deviation

5.4.3 Identification of the right stage of microspores and their *in vitro* development: Embryogenic microspores at early uninucleate to early binucleate are recognized by a large vacuole compartment and by the centrally positioned nucleus, compared to non embryonic ones. Besides, the viable and right stage microspores were identified by their blue exine (Fig.19e) instead of red or black of the old and non embryonic ones (Fig.19f). When the majority (approximately 50 %) of the isolated microspores consisted of this stage, it gets swollen and in most cases star-like structures (SLS) (Fig. 20a arrow) were observed while they are still stored at

cold pre-treatment for one to three days either in the starvation or normal nutrient media. When these viable and swollen microspores were transferred to nutrient media and culture incubation (25±1°C), 10-40 % of the microspores remained swollen while most of the non embryonic microspores shrivelled and degenerated with in two days. The un-pre-treated microspores mostly proceed to a normal gametophytic development within 1-2 days of culture and are filled with starch grains and become darker and further growth of pollen tubes were also observed *in vitro.* The responding microspores began division (starting 24-72 h) after they were cultured in the nutrient media and continued growing to sporophytic stage within one week of culture. Asymmetric divisions (Fig. 20b) were observed 3-5 days after culture (dac) forming two vegetative like nuclear cells of similar size. This is in contrast to the normal asymmetric division that generates vegetative and generative nucleus *in vivo*. The dividing microspores either develop oily structure (Fig 20c arrow) or further divided and formed multi-cellular (4-16) structures (Fig. 20d) after 7 days of plating. However, normal development did not continued due to the disturbance of the unknown toxic substance which exudate out of the germ pores of the dead and degenerated microspores, thus, resulted formation of sand like partiticles and polluted the culture dish (Fig. 20e). The exine of the multicellular cells broke (Fig. 20f arrow) to form embryo like structure (ELS) (Fig. 20g arrows) at the ninth to twelvth day. These were selectively transferred to solid medium after two-three weeks and they grew to 2 mm size microcalli (Fig. 20h) after a total of 4 weeks of culture.



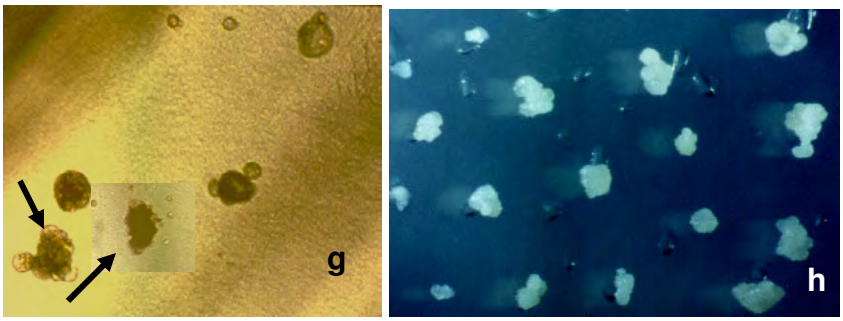


Fig. 20 Sporophytic development of microspores **a**) star like structures **b)** microspore at symmetrical division **c)** microspores developing oily like structure after symmetrical division showing interruption of further growth **d)** microspores division at 4 (red arrow) to 16 cells (green arrow) stage **e)** sand like structure excreted from the dead and degenerated microspores **f)** microspores breaking their exine **g)** embryo like structures (ELS) obtained after 2 weeks **h)** calli obtained from haploid microspores 4 weeks after culture.

5.4.4 Stress pre-treatment: Microspores division and sporophytic development were observed only in the stress pre-treatment (both temperature and starvation media) but not in the control (Table 26). A symmetric microspores division was obtained from varieties Alba and DZ-01-974 at pre-treatment 1 and 4 respectively using starvation medium containing 0.4 M milibiose. Most of the microspores switched to sporophytic development in the approximately 40 % of the isolated microspores in starvation medium. They were swollen and started sporophytic division after 3-5 days in the nutrient medium and the rest 10-30 % of the microspores continued multi-cellular sporophytic division upto the seventh day and break their exine on the 9^{th} day.

There was remarkable variation among the treatments when variety DZ-01-974 (Table 26) was used. The highest percent (0.56 and 0.38 %) of ELS were obtained from pre-treatment 4 using mannitol and milibiose starvation media, respectively. Two-fold increase was observed in the percent of ELS produced from this pre-treatment than pre treatment 5 (without starvation). The response of varieties Alba and DZ-01-974 vary for the two starvation media containing mannitol and melibiose. The variety Alba preferred milibiose than mannitol whereas, variety DZ-01-974 preferred mannitol (Table 26). In general, pre-treatment 4 (see Table 26) were found to be the best for both genotypes and were selected for the next experiments.

Table 26. Effect of panicle and microspores pre-treatment of variety DZ-01-974 cultured using K99M2 on the percent of ELS formation.

Trt No.	Pre-treatment of panicle and culture temperature	Additional Pre-treatment	Duration (days)	Total No. of ms isolated	Total No of viable ms	% of mcc	% of ELS
1	Heat shock at 32 °C	Melibiose at 4°C	1½	100000	8375	4.93	3.29
		Mannitol at 4 °C	1½	350000	9166	4.0	2.91
2	Panicle 2 weeks at 4°C	--	1-3	100000	8500	1.9	0.51
3	No treatment	Mannitol at 4°C	1½	100000	1499	1.49	1.21
		Melibiose at 4°C	1½	100000			
4	Panicle 2 weeks, 4°C	Mannitol at 4°C	1-3	10000	12044	7.1	4.27
	Panicle 2 weeks, 4°C	Melibiose at 4°C	1-3	25000	13206	6.7	5.54
5	Panicle, 2-3 weeks at 4°C	Nutrient medium at 4°C	-	1000000	13561	2.9	0.57
6	Control, freshly isolated ı	Nutrient medium at 25°C	-	1000000	22920	0.0	0.19

NB: mcc-multicellular cells, ELS-embryo like sturctures

5.4.5 Microspore culture media: In the preliminary observation trial both MS and N6 medium were used but the number of microspors showing the first symmetric division were very few. Thus, in the later experiments only K99C4B was used due to its better effect compared to MS and N6. K99C4B liquid medium was used for barley microspores culture especially for cv. Igri (Hönicka, 2001). However, the tef microspores did not grow in this medium as barley (cultivar Igri) and wheat. So, various modifications were attempted such as reduction of the glutamine level by half (from 7 to 3.5 mM) addition of 0.1 µM 2, 4-D in both liquid and solid media. These medium was found to increase the number of dividing microspores and ELS by two-three fold in the liquid medium as well as in the solid medium for variety, DZ-01-974. Besides, none of the microspores cultured in the medium devoid of the growth hormone, 2, 4-D was able to break their exine after multicellular cell formation. Thus, both media were supplemented with 1 µM 2, 4-D. However, the number of ELS formed in liquid medium was much higher than the solid medium. Therefore, the two liquid media K99C4B and K99M2 were compared only in liquid state in experiments to follow.

Table 27. Effect of microspores culture media and feeder (from barley microspores) on the formation of multicellular cells (mcc) and microspores derived ELS for the two tef genotypes.

Genotypes	Pre-treatment	Feeder cells	Media	mcc	ELS

				K99C4B	10.5	9.50
DZ-01-974	Treatment mannitol	4	Igri microspores	K99-M2	120.1	251.1
				K99C4B	6.8	24.0
			Ovaries	K99-M2	9.6	23.8
				K99C4B	5.3	23.4
Alba	Treatment melibiose	4	Igri microspores	K99-M2	77.0	192**.0**
			Ovaries	K99C4B	1.56	34.1
				K99-M2	5.3	23.5

Data were collected 30 days after microspores culture initiation and are the mean of three replications, NB., mcc=multicellular cells, ELS=embryo like structures

The rate of ELS formation between the two nutrient media using pre-treatment 4 in combination with the feeder cells were compared between variety DZ-01-974 and Alba. The result showed variation in the number of ELS formed between the two genotypes (Table 27). The improved variety out perform both in multicellular cells and ELS formation using K99M2 medium. However, in both cases K99M2 medium showed better result for both varieties (Table 27).

5.4.6 Co-culture and feeder effect: The number of dividing cells did not increase in the presence or absence of ovaries co-cultured with the microspores. Rather, the co-cultured ovaries enlarged till the fifth day and collapsed gradually. This might indicate that the cultured microspores did not benefit by the presence of the co-cultured ovaries. The effect of co-cultured ovaries and feeder cells was compared using DZ-01-974 in K99M2 medium. As Table 28 shows, significant variation was observed between the four co-culture treatments.

Table 28. Effect of feeding on the percent of division, SLS and ELS formation of the cultured microspore of variety, DZ-01-974.

No.	Treatments of Feeding	Total No. of ms	% of SLS	% of division	% of ELS
1	No feeder and no ovaries	13098	0.01	8.0	0.27
2	Ovary co-culture	16024	0.58	9.1	1.65
3	Feeder, immature embryo calli of tef	11032	0.11	2.1	2.43

4	Feeder, one week old sporophytic microspores of var. Igri	21099	16.8	29.7	9.36

NB. SLS- star like structure, ELS, embryo like structures

Co-culture with 1 µl of one week old sporophytic microspores suspension of barley showed high (7.3 %) development ELS formation and was followed by ovaries. However, the appropriate concentration of the feeder cells needs to be further evaluated in order to reduce competition between the two genotype cells and increase the number of induced ELS. Further investigation of the feeder cells effect was also compared using one week old tef somatic embryonic calli. However, there was very few (0.05 %) ELS formed from this attempt. The number of multicellular cells and induced ELS was increased in the co-cultured barley microspores used as a feeder. As a result, repeated experiments were done using the improved technique to obtain microcalli and calli to regenerate haploid plant from tef.

5.4.7 Problem associated with further growth of dividing cells:

Although the dividing cells induced using the highly improved technique were interesting, further growth of the microspores was blocked after 15 days and those dividing microspores turned to black. This was because of some black grain excaudate from the germ pores of the dead and degenerated cells that accumulated as sand (Fig. 20e) and affect the growing microspores in the culture medium. The toxic substance surrounded the living dividing cells and blocked further growth. Moreover, some microspores developed oily structure (Fig. 20c) after the first cell division. This also inhibited further division to form multicellular cells (mcc) and ELS formation. This substance which is excreted from the degenerated microspores in culture could be poisonous solution (Hönicka, per. com)**.** In order to improve the growth of the responding microspores freshly prepared medium was added into the culture dish after the old medium was sucked every other day for 7-10 days and also every three day for three to four weeks. Therefore, two purification techniques were tested to isolate the viable and responsive stage of microspores from the matured, dead and collapsed microspores before culture initiation (see 5.4.2.1).

5.4.8 Genotypes: **Among the four genotypes tested varieties DZ-01-974 and Alba were found to be responsive whereas, Fesho and DZ-CR-37 were relatively recalcitrant for microspores culture.**

5.4.9 Regeneration: Various regeneration media were used in order to regenerate those calli induced from microspores culture. Sub culturing to fresh media was attempted for six months. However, none were regenerated to green plant except one albino plant that died before being transferred to pot soil.

5.5 Discussion

5.5.1 Factors affecting androgenesis in tef: There were combinations of factors that affect the response of anther and microspores cultures in tef. These include the growth condition of the donor plant, harvesting stage of panicles, growth stage of anthers/ms, pre-treatment, microspores density, effect of co-culture and feeder, genotypes and media. These main limiting factors which were reported for microspores culture study of the major cereals (Datta, 2001) are similar for tef.

5.5.1.1 Growth condition of the pollen donor plants: The physiological conditions under which donor plants are grown can significantly affect subsequent responses to the isolated microspores (Chen, 1986, Chen and Li, 1978 in rice, Gu et al., 1978 in maize, Hönicka, 2001 in wheat and barley). According to these reports, the induction frequency of any genotype usually depended on the growing season and whether the plants are grown in the field or in the green house. For instance, among the factors which affect rice pollen culture was temperature (Chen, 1986). Since the study materials of tef were obtained continually from the green house grown plants, almost all tef varieties showed abnormality in the form of plant growth and panicle maturity (late heading). This could be due to the insufficient light during winter (8 hrs dim light) and the long day (16 hrs) effect as well as the high humid condition (70 %) of the green house. Poor spikelet formation per panicle in winter, abnormal panicle morphology and distorted maturity and sterility in summer grown plants were the main obstacle in these experiments especially when using the late maturing varieties. Microspores cultured from winter showed better culture response than the summer grown plants. Relatively Fesho grew normal through out the year and samples were taken from this variety for a longer time.

Generally, normal growth of the donor plant is a prerequisite for positive response for both anther and microspores culture, with stages at the booting of spikes for barley, wheat and rice but at early heading to tef.

During the induction processes of microspores that developed from gametophytic to sporophytic stage, there are two sensitive periods to be considered and these include developmental process of young anthers before the start of meiosis and the tetrad at early uninucleate microspores stage. During these periods the influence of temperature, weak or strong light and too little daylight may affect (Chen, 1986). The observations noted on tef were in agreement with rice panicle growth for anther culture (Chen, 1986).

The better response of winter grown plants over the summer may be due to the slow growth of panicles (anthers and microspores) in the short day light period which attributed with the low temperature of Hamburg, so that ample microspores can be isolated at the right stage especially from the intermediate-late variety DZ-01-354. The daylight period in Ethiopian environment was nearly similar through out the year and lasts only for the maximum of 12 hrs. The relatively mild growing temperature during heading period of tef at the main rainy season of Ethiopia could be the most appropriate to the normal development of anthers and microspores. The low temperature could slow the development of microspores in the anther and this was also highly supported by the (4°C) pre-treatments of the sampled panicle to prevent the microspores development to gametophytic path way. The abnormal growth of the tef plants/panicles throughout the research period in Hamburg made these experiment much difficult than expected especially due to the low number of isolated microspores. Generally, low or high temperature is critical for the donor plant growth. For instance a beneficial embryogenic effect was obtained when plants were grown at low temperature for *Brassica napus* at 10°C/day and 5°C/night. Besides, as previously stated the effect of light intensity and photoperiod are also important factors for androgenesis (Keller et al., 1987).

5.5.1.2 The developmental stage of microspores: Large numbers of studies have demonstrated that microspores will respond only at a specific developmental stages that range from the early uninucleate to the early binucleate stages in all species (Keller et al., 1987, Chen, 1986, Bedinger, 1992, Dunwell, 1992). These stages are clearly seen in tef microspores and are found mainly from the normally grown panicles of the tested varieties when 1/2 - 1/3 of its panicle emerged out of the sheath. According to our result, using variety DZ-01-974, the middle and

small anthers contained early to mid uninucleate stage of microspores were considered as an appropriate stage for anther and microspores androgenesis in tef very similar to what is reported for most cereals (Datta, 2001).

The highly vacuolated microspores which contain nucleus at the central position (early uninucleate stage) moved towards the pore and reached at the opposite direction of the pore (late uninucleate stage) where both stages contain blue exine and are thus considered as viable and at the right stage for tef microspores. Enlargement and sporophytic division was obtained at about 70 % the cultured cells when the other preconditions, such as pre treatment and media combinations were fulfilled. Division was obtained from both middle and small sized green anthers with varying proportion. The star like structures and ELS formed in tef cultures resembled that of reported in barley (Hoekstra et. al., 1992), maize (Wan and Widholm, 1992) and wheat (Hönicka, 2001) microspores cultures. Microspores with collapsed cytoplasm and red exine are non viable and also shrinked microspores types are considered as non-viable. Besides, matured pollen of tef can easily be recognized by their cytoplasm which is fully filled with starch grains that are darkened. The very small microspores with empty cytoplasm and without pore also remained unchanged for several weeks and later collapsed and died. The later three stages created problems when cultured with the viable right stage microspores. Purification of these stage microspores was very important to avoid the destruction of viable and dividing microspores. Comparative studies have indicated in rice callus induction frequency that the highest embryos were obtained when anthers containing microspores with mid uninucleate stage (Chen, 1986). Similar report was obtained from wheat and barley anther culture studies (Hassawi et al., 1990; Hönicka, 2001). However, there are few exceptions in wheat anther culture where plantlets were obtained from anthers at pollen mother cell stages (He and Ouyang, 1984). However, in this case also the initiation of embryogenesis may start after the very immature anthers completed the developmental growth in *in vitro* culture and proceeded to sporophytic path way.

Tavassoli (1986) also reported that pollen mother cells (PMC) stage of tef was found when 1/3 of the panicles emerge out of the flag leaf sheath. This leads to the fact that the first pollen mitosis (uninucleate stage) of tef can be reached when 1/3 to 2/3 of the panicle emerge out of the flag leaf sheath which is in contrast to wheat, barley and rice where early to late uninucleate stage are normally found from the booting stage, before the spikes or tassel emerge from the sheath. The

toxic substance released from the dead microspores affected microspores culture in tef. This phenomenon was repeatedly reported by several authors in cereal microspores embryogenesis. According to Hönicka (2001) purification of these harmful microspores prior to culture initiation was advantageous.

5.5.1.3 Effect of pre-treatment: The effect of stress pre-treatment on the induction of microspores embryogenesis was evaluated for tef microspore culture. Stress has been proposed to be essential to switch the genetic program of the microspores from gametophytic to sporophytic development pathway (Jähne and Lörz, 1995; Touraev et al., 1997, Indrianto et al., 1999, Custers et al., 1994, Raina and Irfan, 1998). Ouyang (1986) and recently Hönicka (2001) demonstrated the development of plants and ELS from non-pretreated wheat anther and wheat and barley microspores cultures, respectively. Hönicka (2001) stated that stress factors like, wounding of anthers, spike sterilization and microspores isolation procedure may probably activating genes involved in the microspores embryogenesis of the unpretreated microspores but to a lesser extent than starvation and temperature pre-treatments. Stress treatment, such as; starvation cause DNA replication in the vegetative cell of the developing pollen grain and specific mRNAs are synthesized and change protein kinease activity (Touraev et al., 1997). Canola and brassica microspores subjected to 32.5 and 33°C for 24h and 8h exhibited 21 % and 31 % of embryo development, respectively. After high temperature shock microspores begin to divide in a symmetrical manner. A loss of cellular polarity, an increased symmetry occurs after 24 h of treatment.

In tef non pre-treated microspores of all genotypes were developed to normal gametophytic pollen grain formation. Another structural change associated to high temperature, treatment is the appearance of cytoplasmic granules. These granules appear to be the heat shock proteins. These proteins can involve in the de-differentiation process and may prevent the synthesis of regulatory proteins necessary for pollen development. These structures also observed in the heat pre-treated microspores of tef. However, these features are not absolutely markers of embryo development. In tobacco sucrose and nitrogen starvation of immature bi-cellular pollen grains induce the formation of embryogenic pollen grain after transfer to a simple medium containing sucrose and nitrogen (Tauraev et al., 1996b). A combination of starvation with heat shock induces embryogenesis in nearly all of the living microspores in indica rice and tobacco (Raina and Irfan,

1998, Tauraev et al., 1996b). After the release from the tetrad, microspores are surrounded by a thin walled pri-exine and the nucleus occupies a central position. Upon stress treatment, isolated microspores swell and their cytoplasm undergoes structural reorganization. The nucleus moves to a more central position and cytoplasmic strands are formed that pass through the vacuole and connect the perinuclear cytoplasm with the subcortical cytoplasm. These features have been observed in tef even while microspores are still in the starvation medium. This was reported by many authors in several species including wheat, rice and tobacco. Changes in temperature, from the donor plant grown at 17°C to microspores culture at 26°C might also represent an additional stress (Hönicka, 2001) in wheat and barley microspores cultures. In tef the growth temperature of the donor plants (26°C) was the same as the microspores culture temperature. Therefore, the freshly isolated microspores couldn't switch to sporophytic development as did for wheat and barley microspores.

Varietal response varied for identical pre-treatments for cell division. For instance, in variety Alba panicles pre-treated at 33°C for 1-3 days showed very low response compared to variety DZ-01-974. On the other hand panicles of genotypes Fesho, DZ-CR-37 and DZ-01-196 stored at 4°C for 2-3 weeks and co-cultured with igri microspores exhibited different response, in that only DZ-01-196's microspores divided to multicellular cells.

Thus, interruption of the gametophytic development of microspores by a combination of stress treatment (temperature shock of panicle at 4°C for 2-3 weeks as well as sugar starvation applied to the isolated microspores containing high levels of mannitol together with the feeding effect) may be a strong influence to switch to the sporophytic pathway of development. Recently Raina and Irfan (1998) demonstrated high efficiency embryogenesis and plantlet regeneration from isolated microspores of indica rice by heat shock (33°C) and starvation of microspores (0.4 M mannitol) for four days. Similar result was also reported by Touraev et. al., (1996b) from *nicotiana tabacum* microspores and by Heberle-Bors (1998) and Touraev et.al., (1996a) from wheat microspores cultures. Mannitol stress pre-treatment is widely used for isolated microspores culture of barley. Tef microspores subjected to a mannitol pre-treatment for 1-3 days at high temperature (26°C) inhibited the growth of the microspores to sporophytic development and died after few days of culture. Favourable factors for sporophytic pathway may include optimal physical condition such as osmolality and proper ingredients in the media

including carbohydrate, plant hormones, minerals nutrients, vitamins etc (Mejza et al., 1993). If conditions are sub optimal many competent microspores may either fail to divide or terminate their divisions by aborting their embryonic process with a substantial reduction in yields of microspores derived plants (Zhou et. al., 1991; Zheng and Konzak, 1999; Zheng et al., 2001). This might be one of the reasons for the death of the embryonic tef microspores that were pre-treated in starvation medium and incubated at 25°C instead of 4°C. Those microspores which were stored for 2-3 weeks at 4°C were already switched to sporophytic development and may require the nutrient media rather than the starvation at the optimal temperature (25°C) for microspores induction to further induce ELS. In contrast, the microspores may improve their sporophytic development by further treating them in starvation medium but at 4°C. At 4°C the metabolic activities of cells are slowed compared to hot conditions. Pretreatment is also known in its effect to increase the frequency of spontaneous chromosome doubling in wheat anther culture (Ouyang, 1986).

5.5.1.4 Culture media composition: After the repeated termination of division, we decided to focus on the effect of media composition. Since at the beginning only two media, K99 (Kumlehn unpublished data) and N6 were compared using many varieties including Fesho and DZ-01-974. MS was also tested in anther culture studies and there were no big differences with K99 in the formation of multicellular cells. So, only K99 was taken to run most of the experiments by considering the importance of growth regulators. According to the report of Datta (2001), in the majority cases, auxin or cytokinin has been required as a supplement in the anther culture medium. Hoekstra et al., (1996) also showed the effect of 2, 4-D on microspore culture of barley. However, calli/embryos have also been induced in culture medium without growth regulators, e.g. in maize (Nitsch, 1981; Tsay et al., 1986; Kuo et. al., 1994), wheat (Chu et al., 1973) and rice, or with cytokinine alone; e.g. in wild *Hordeum* species and cultivated barely (Olsen, 1987; Kihara et al., 1994). K99 medium was also used without auxin but with little cytokinine (BAP) supplement. Hönicka (2001) also showed the effect of growth hormones (auxin-cytokinin combination) on the increase of green plants per 100 anther compared to media devoid of hormones on wheat and barley microspores cultures. Kivihurhu and Tauriainen (1999) also studied the effect of the two hormone combinations and showed that high auxin concentration of 6.9 mg/l (2, 4-D) promoting direct embryo initiation in *A sativa* although embryos were also

initiated without growth regulators.

So attempts to improve the composition of K99C4B medium by reducing the glutamine concentration, with and without 2, 4-D and with the removal of the BAP in the half concentration of the basic medium salt were tested. Low basic media salts and nitrogen levels for immature explants were believed to show much better results for androgenic results (Kumlehn personal communication) and K99C4B has low concentration of nitrogen compared to MS and N6. The half concentrated medium with the addition of sucrose instead of maltose did not show better results. Therefore, only medium that contained low level of glutamine and supplemented with 0.1 µM 2, 4-D, (named K99M2) was compared with the original medium. Interestingly, microspores can break their exine only in 2, 4-D containing media after 10-13 days and the number of multicellular cells which formed ELS increased by 10 fold at K99M2 medium, with half glutamine concentration after 3 weeks of culture is reported for the first time**.** This new combination improved the growth of the multicellular cells to ELS formation for the first time and high number of ELS was also obtained from this medium. So, auxin was considered as one of the essential factor to initiate callus induction in tef haplodizaiton studies. The effect of auxin (2, 4-D) was also clearly observed in tef somatic embryogenesis studies derived from immature embryo cultures (Chapter 5). None of the medium devoid of the growth hormone showed somatic embryogenesis in tef studied here.

Similarly none of the microspores from the cultured anthers without the growth hormones developed to induce globular embryos (see part I of this chapter). These factors were repeatedly reported in several studies of anther and microspores culture of cereal crops. Microspores of tef were cultured at 25±1^{o}C incubation chamber and dividing cells can be achieved with in 24 h if the pre-treatment is adequate. Using shaker and addition of fresh medium every 3 days and or at 3, 5, 7, and 10 days or supplementing with some vitamins did not show any improvement to further division and breaking of exine in all tested varieties.

5.5.1.5 Effect of ovary co-culture and feeder cells: As reported by many authors viable ovaries to culture media are beneficial for microspores embryogenesis but only to the responsive genotypes. Furthermore, when microspores that were isolated from the recalcitrant wheat genotypes were cultured with the ovary conditioning medium (five live ovaries, as a co-culture),

yields of microspores embryoides showed over a 100 fold over the control (Zheng et al., 2001). Co-culture of live ovaries also enabled to develop the highest number of ELS per 100 microspores for wheat and barley microspores culture when the microspores density was least, 75 per ml (Hönicka, 2001). However, the feeding effect of the co-cultured ovaries of tef and wheat, on tef microspores culture was not seen, except the enlargement of the ovaries up to their maximum size till the fifth date of culture. There was no significant difference in the dividing cells of tef even when the number of ovaries per ml medium was increased (up to 30) and ovary conditioned media was used. Zheng et al., (2001) clearly showed the effect of live ovaries and medium pre-conditioned by ovaries on microspores embryogenesis of common wheat. According to these authors 4-5 fold increase in live ovaries, resulted in more than 100 percent increase over the control. However, in tef co-culture treatments such results were not obtained. The growth of the co-cultured ovaries in culture might indicate that the ovaries may benefit from the nutrients and used the growth hormone for them instead of feeding the microspores. Besides the ovary conditioning medium (OVCM) prepared by using the culture medium of Zheng et al., (2001) may not be suitable to tef microspores culture.

The induction of ELS of tef microspores was highly improved by using the fast growing (1 week old) sporophytic microspores of barley, cv. igri as a feeder cells. Beyond the feeding effect it was also reported that stimuli can be transferred from the growing embryos to the recalcitrant genotypes by co-culture (Kumlehn personal communication). The co-cultured tef microspores on both liquid and solid media using barley microspores suspension and tef somatic cells derived from immature embryos, showed microspores division and multicellular cell formation for variety DZ-01-974 compared to none co-cultured dishes. However, the quantity and quality of the feeder cells should be taken into consideration to obtain better result in microspores culture. This will help to prevent competition for the nutrient medium and growth hormones. Experiments were repeated with the co-culture feeder of barley microspores and results were much better. Therefore, it is important to state that tef microspores should be co-cultured with a feeder cells especially the fast dividing cells from a responsive genotypes in the culture protocol combined with the suitable pre-treatment of panicle and isolated microspores other than the ovaries. One week old embryogenic microspores of barley cv. igri also reported to improve the culture conditions of wheat zygotic embryo development (Kumlehn et al., 1998). In co-culture (feeding) signals can be exchanged from the fast growing embryonic cells to potential cells of

other species. However, intensive study should be carried out to find the appropriate proportion of the feeder cells with the cultured microspores of tef to achieve high cell division and to avoid negative competitions between the cells. In addition, other feeder source other than cv. igri, should be studied which can easily be available in Ethiopia.

5.5.2 Improving the microspore isolation and purification techniques: In cereals, the use of blending in microspore isolation has been widely used. e.g. for wheat (Hu and Kasha, 1997, Hönicka, 2001); barley (Mordhorst and Lörz, 1993, Jähne and Lörz., 1995, Hönicka, 2001) and maize (Nägeli, 1998) also vortexing and mixing methods are still used (Indrianto et. al., 1999). The development of microspores isolation technique for tef took much of the time during the first six months and several improved techniques were attempted. The changes in the sample preparation, repetition in the first part of centrifugation and increasing the time for the second gradient centrifugation made a significant improvement and as high as approximately 500,000 to 1,000000 viable microspores were collected in the interphase band between mannitol and maltose solutions. Spikelets were prefered to be collected instead of slice panicle cuts for the preparation of samples. In slice cutting of panicles not more than 10,000 microspores were collected. However, the improved method is relatively time consuming and tedious but obviously effective for increasing the number of microspores. The second modification was to increase the time of the second centrifugation from 10 to 15 minutes and the last minor modification was to use a 30 µm mesh instead of 50 since tef microspores are very small.

The separation of viable and dead microspores has been used to improve microspores cultures (Kyo and Harada, 1985; Mordhorst and Lörz, 1993; Hönicka, 2001). Purification of the viable microspores from the dead ones was also challenging and repeated attempts were made. The two purification techniques, using percoll and maltose solutions to a gradient centrifugation in high speed were compared. The percoll method was not appropriate because, many microspores were

lost during the washing process using mannitol. This process needs extra time and many microspores are lost throughout the process. Later, we attempted using only 0.4 M mannitol and 19 % maltose in-between the tef microspores in the medium and gradient centrifugation for 15 minutes. The result obtained from this work indicates that the use of 19 % maltose allows higher purification efficiency than the percoll method. Besides, this method did not consume too much time.

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Chapter 6

In vitro Gynogenesis in Tef

6.1. Materials and Methods

6.1.1 Plant Materials: DZ-01-196, DZ-CR-37, Fesho and *E.mexicana* were used for the study of *in vitro* gynogenesis in tef. Plant growth, sterilization, and excision of unpollinated pistils were as described in chapter 3. The study was carried out in Hamburg, Germany.

6.1.2 Suitable explant type: The following three explant types namely excised pistils with or without co-cultured anthers; panicle segments and detached spikelets were used**.** Young panicles, which emerged 14-17 cm (Fig. 16) from the flag leaf, except stated other wise were used to excise unpollinated ovaries and panicle segments in many of the experiments.

6.1.3. **Developmental stages of explants:** Pistils at four stages namely; (S) small, (immature ovary, (pre meiosis), (I) intermediate, (2-3d before pollination (uni-binucleate) and (L) large, (one day before pollination (mid-late binucleate) and very large (VL) trinucleate stages of anther development (Fig. 21a) were cultured separately or co-cultured with anthers. For panicle segment culture (Fig. 21b), various developmental stages of emerged panicles with, 0-5, 6-9, 10-13, 14-17 and 18-20 cm out of the sheath were compared.

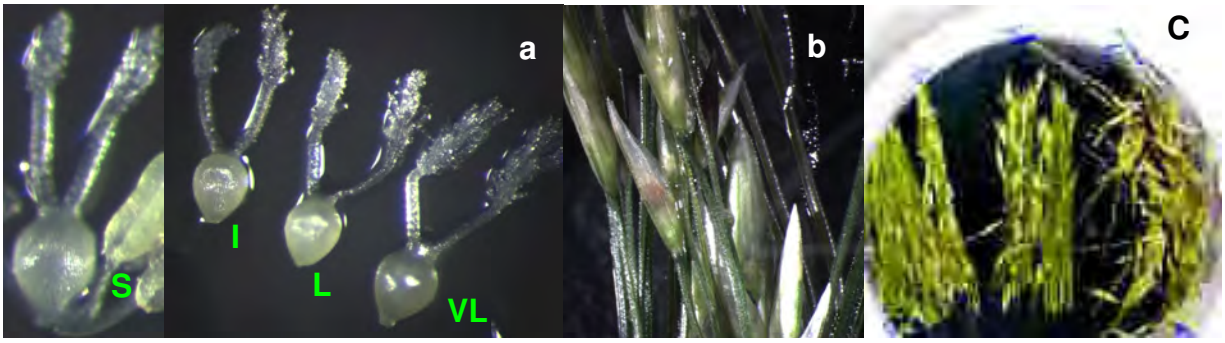

Fig. 21 Developmental stages of cultured explants **(a)** unpollinated isolated pistils at various stages, small. I, intermediate, L, large, VL, very large (clear variation in the length of stigma), **b)** cultured panicle segment, sample taken from 1/3 of panicle emerged out of the sheath **c)** panicle segments cultured in three positions bottom, intermediate and top (from left to right)

6.1.4 Appropriate panicle part: Only explants from panicle stage of 14-17 cm were used to determine the appropriate panicle part. The panicles were divided into three parts, bottom, intermediate and top, each approximately 5 cm (Fig. 21c) and cultured in separate petri dishes.

6.1.5 Effect of pre-treatment duration: Freshly isolated pistils were cultured on the media and incubated under heat (32°C) or cold (4°C) treatments for 1-3 days before being transferred to the culture temperature at 26°C in dark or 22±2°C under light (16 h) chamber. For panicle segment culture; freshly harvested panicles were stored at 4°C for 12, 24, 36 and 72 h and compared with the control (no pre-treatment) and cultured under both light and dark conditions.

6.1.6 Media: MS (Murashige and Skoog, 1962) basal medium supplemented with 10 µM 2, 4-D was used for the tef varieties while modified L3 basal medium (Jähne et. al., 1991) was used for the wild species. Media was solidified with 3 % Gelrite and the pH was adjusted to 5.8. To standardize the culture protocol, only variety DZ-01-196 was used. Experiments were under taken one after the other and, the best optimized condition was used in the subsequent

experiments. Finally, the responses of the other genotypes were tested using the standardized conditions that were based on variety DZ-01-196. The following four independent experiments were conducted.

6.1.6.1 Effect of 2, 4-D concentration: In all the above experiments medium was supplemented with 10 µM 2, 4-D. The concentration of 2, 4-D was optimized by doubling the levels till the highest embryonic tissue (ET) formation. The concentration tested were 10, 20, 30, 40 µM using only spikelet and panicle segment cultures.

6.1.7 Culture condition: The response of explants for both culture conditions, a) 26 ±1°C dark and b) 22 ±2°C light (16 h) were compared. The number of cultured explants per petri dish in pistil, spikelet and panicle segments culture was 10-20, 20-30 and 50-100 respectively. Sealed petri dishes were incubated at 22±2°C with 16 hours of light per day for15-30 days till the embryonic tissues (ETs) are induced from the responsive pistils. The ETs, were left attached with the florets or excised and were later transferred to regeneration medium MSSI (MS medium with 0.1 µM 2, 4-D. After two weeks explants were transferred to Magenta vessel filled with MS but devoid of exogenous plant growth hormones. The responses of all genotype for panicle segment culture under the optimum culture protocol developed for variety DZ-01-196 were compared.

6.1.8 Flow cytometry analysis: The better grown regenerants after 4 weeks in the regeneration medium were transferred to pots containing peat soil. The polyploidy level of these regenerated plantlets were analysed using a Partec CA-II flow cytometer (Partec, Ploidy Analyser, PA GMBH, Otto-Hahn Strasse 32, D-48161 Münster, Germany). Samples were taken from leaves of 4-7 weeks old regenerants. Leaves were chopped using Razor blades in a 250 µl of ice cold commercial cystain, UW ploidy DAPI staining solution in a petri dish. One ml of the same staining solution was added to the chopped tissue and that was filtered through 15 μm nylon screen to remove the debris. The suspension containing nuclei was used for analysis immediately. The cytometer was adjusted prior to analysis using the control and checked repeatedly for standard peak, of tetraploid (2C) seed derived plants. Three samples were taken form each of the 182 regenerants and 50 samples of the control from each variety. Each sample contained at least 5000 nuclei for measurement.

6.1.9 Data collection and analysis: The experiments were replicated 5-10 times and repeated three times. The contaminated petri dishes were removed and only the uncontaminated dishes were counted. Percentage of callus induction, embryonic tissue formation and plant regeneration were calculated from the total number of explants cultured.

6.2 Results

6.2.1 Pistil culture: The response of unpollinated excised pistils without anthers attachment is illustrated in Fig. 22. Preliminary experiments revealed that culture without growth regulators and cultures containing half concentrated MS medium components failed to result in pistil enlargement. However, pistils were enlarged (Fig. 22b) both in light and dark culture conditions pre-treated either in heat (32°C) or cold (4°C) treatments. The enlarged pistils turned to green three days after incubation when cultured in light and almost immediately when transferred from the dark (5 days) to the light chamber. Enlargement continued in liquid as well as solid medium for about 5 days of culture irrespective of anthers attachment and was independent of the genotypes used. However, most of the cultured pistils gradually collapsed after five days (Fig. 22c). Ovules dissected from these pistils also collapsed. However, few percent of callus induction was obtained from the intermediate stage ovaries of tef and larger ovaries of the wild species. The effect of the developmental stage of the cultured pistils and duration of pre-treatment on enlargement of pistils and callus induction is shown in Table 29. Except the small pistils the intermediate and large pistil showed no variation for enlargement but showed variation for callus induction. Only the intermediate stage of pistils induced callus for DZ-01-196 and the larger ones (one day before pollination or trinucleate stage) for the wild species.

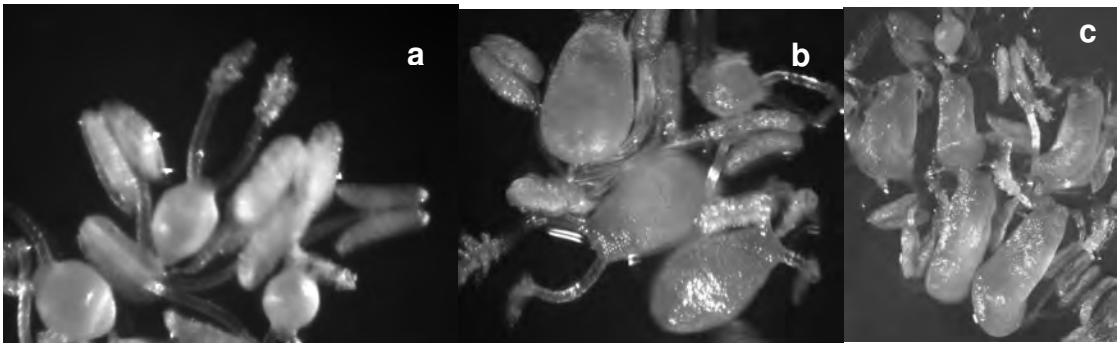

Fig. 22 Development of unpollinated isolated pistils in culture, co-cultured with the respective anthers **a)** freshly cultured S size pistils **b)** enlargement of pistils after 5 days and **c)** after 7 days of culture.

Very large (VL) pistils did not respond to this culture condition thus, were omitted from further experimentation. Up to 3.4 % of callus induction was obtained from intermediate size of tef variety, DZ-01-196 and 12 % from the larger pistils of *E.mexicana* (Table 29). Fresh materials were highly responsive in both species (Table 29). The calli derived from the unfertilized pistils of tef variety, DZ-01-196 were white and amorphous type (Fig. 23b) and regenerated to roots only (Fig. 23c). There was no variation in ovaries enlargement between Fesho and DZ-01-196. In contrast, freshly cultured, large pistils of *E .mexicana* induced small friable proliferating callus at the micropylar end of the ovaries (Fig. 23a) and regenerated to 6 green plantlets in the regeneration medium. However, 5 were accidentally contaminated *in vitro* and one was grown to normal, fertile plant. The ploidy analysis of this plant using flow cytometry indicated the same level of ploidy as the donor parent (hexaploid). This could be due to the involvement of the somatic tissues of the ovary in the formation of callus induction and embryogenesis or due to the spontaneous doubling of the haploid chromosomes occurred *in vitro*. L3 medium was not found to be suitable to tef pistil enlargement.

Pistils after 1-2 days of *in vivo* self pollination plus whole panicles with self pollinated florets/ immature caryopsis harvested from the basal spikelets of the top panicle position were cultured using the same medium and culture condition. It was observed that all isolated *in vivo* pollinated ovaries were enlarged for few days but later both ovary and ovules collapsed.

On the other hand some of the immature caryopsis proceeded to normal seed development and were germinated *in vitro* or induced somatic embryos from the white compact callus and germinated in the same medium.

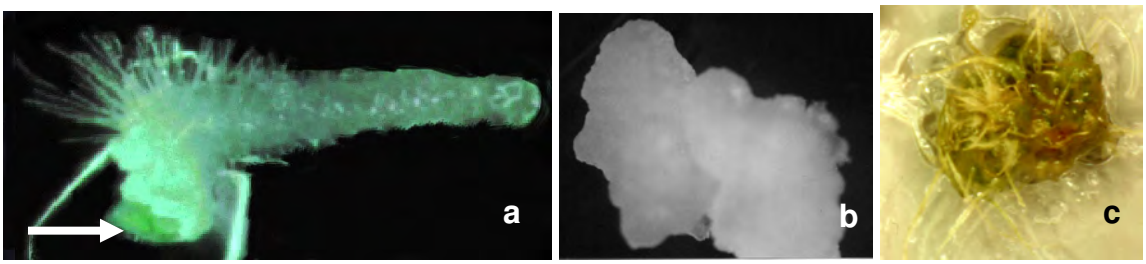

Fig. 23 Development of pistil culture of *Eragrostis* **a)** embryonic callus (arrow) derived from larger ovary of *E.mexicana* at the micropylar end **b)** amorphus callus obtained from intermediate ovary of tef variety, DZ-01-196 and **c)** regeneration of the callus to roots only.

Table 29. Effect of developmental stage and pre-treatment on percent of pistil enlargement, callus induction and regeneration of varieties, DZ-01-196 and *E.mexicana*

Genotypes / *In vitro* response	**DZ-01-196** (Freshly harvested)			**E.*mexicana*** (Freshly harvested			**DZ-01-196** (Intermediate pistils)				**E.*mexicana*** (Large pistils)			
	Developmental stage of pistils						Pre-treatment (4°C) duration (days)							
	Small	Intermediate	Large	Small	Intermediate	Large	Fresh	1-3	4-6	7-9	Fresh	1-3	4-6	7-9
Total pistil cultured	45	58	50	50	65	71	58	50	61	112	50	61	60	112
Total enlarged	5	46	40	12	31	67	46	40	60	56	48	34	58	96
Callus formed	0	2	0	0	1	9	2	0	2	1	6	4	2	2
% of enlargement	11.1	79.3	80	24	47.7	94.4	79.3	80	98.3	50.0	96	57.3	80	85.7
% of callus induction	0	3.4	0.0	0.0	1.54	12.67	3.4	1.0	3.1	0.89	12.0	6.56	3.3	2.0
No. of regenerants	0	2 (roots only)	0.0	0.0	0.0	2 (green plants)	1 (roots only)	0.0	2 (roots only)	0	4 (green plants)	2 (roots only)	0.0	0.0

NB: DZ-01-196 cultured on MS and E.*mexicana on* L3 media

6.2.2 Spikelet and Panicle Segment culture: In most of the tef genotypes and the wild related species (*E.pilosa*, *E.mexicana* and *E.papposa*) which has been used for the different experiments, florets normally contained one pistil having two or three stigmas. However, in this study we observed twin and rarely tripled pistils for variety DZ-01-196 and cultivar Alba both *in vivo* and *in vitro* (Figs. 24a and b) conditions.

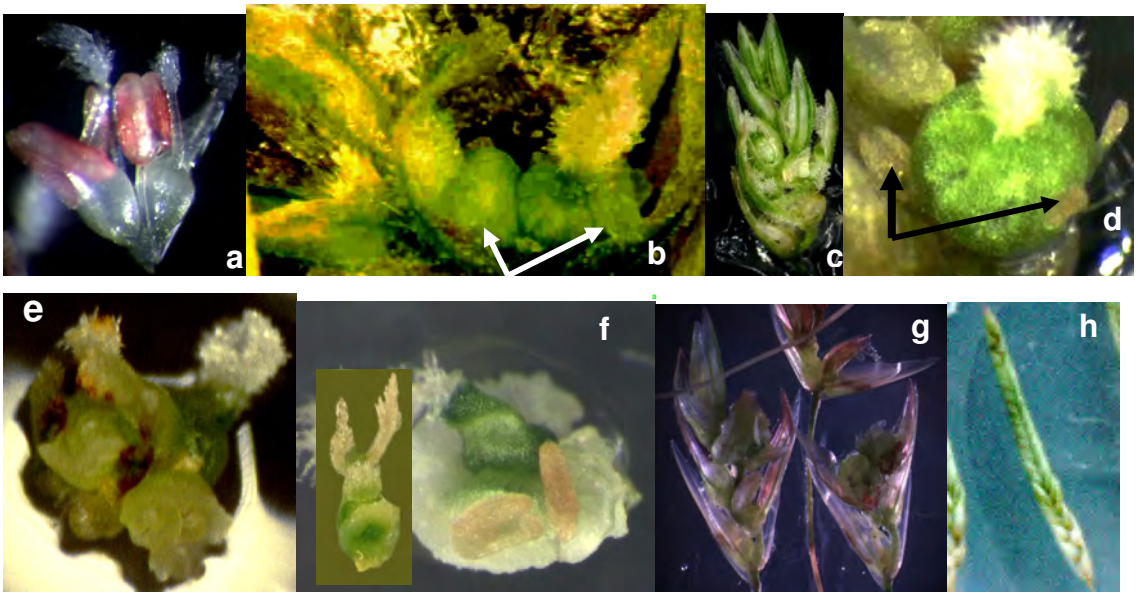

Fig. 24 Gynogenic developments of tef ovaries in panicle segment culture. **a** & **b)** twin ovaries *in vivo* and *in vitro*, respectively **c)** floret growth few days after culture **d)** enlargement of ovary in the panicle segment culture and retarded anther growth (arrows) **e)** embryonic tissue sprouted out of enlarged ovary at micropylar end of ovary **f)** white spongy mass growing around the middle of the enlarged ovary at younger (left) and mature stage (right) **g)** ETs formed at the fourth floret number on a spikelet **h)** secondary floret growth reaching 35 per spikelet.

6.2.3 Development of pistils and induction of embryonic tissues (ETs): In panicle segment culture, floret growth continued and the emergence of new florets (Fig. 24c) appeared few days after culture and the new floret growth continued throughout the culture period until the panicle segment turned to brown. Progressively, the unfertilized intermediate stage ovaries highly enlarged and expanded overtaking the whole position of style and stigma that become brushy, short with the ovary appearing as green pots (Fig. 24d). When compared to the isolated pistil culture this enlargement estimated to be 15 to 20 times larger. Like the isolated pistil culture, the

basal matured unfertilized pistils were fast for swelling but collapsed shortly, while the middle stage ovaries enlarged within 7-10 d. However, the respective three anthers at each floret disintegrated and remained far below the ovary (Fig. 24d arrow). Moreover, in many cases enlarged ovaries formed white spongy mass (Fig. 24e and f), which circled the middle part of the enlarged ovaries. On an average after 10 days of culture, embryonic tissues (ETs) sprouted by splitting and bursting from the enlarged ovaries below the spongy mass (Fig. 24e) and at the micropylar end of the ovary. Sometimes multiple small ovaries also emerged from the single enlarged ovaries, which later sprouted to many embryogenic tissues. Although anthers often degenerated, they rarely grew to the normal mature size and colour; however, these were empty anther (without pollen) and thus failed to proceed with meiosis to form microspores *in vitro*.

No callus phase was observed in any of the embryonic tissue formation of all the varieties tested. A maximum of 5 florets per spikelet were responded for ET formation when panicles were harvested from the vigorously grown main tillers. In contrast to isolated pistil culture here none of the 85 samples of excised ovules were found collapsed and two out of twenty excised ovules (Fig. 25a) cultured on regeneration medium were able to germinate (Fig. 25b) forming the first gynogenic plant.

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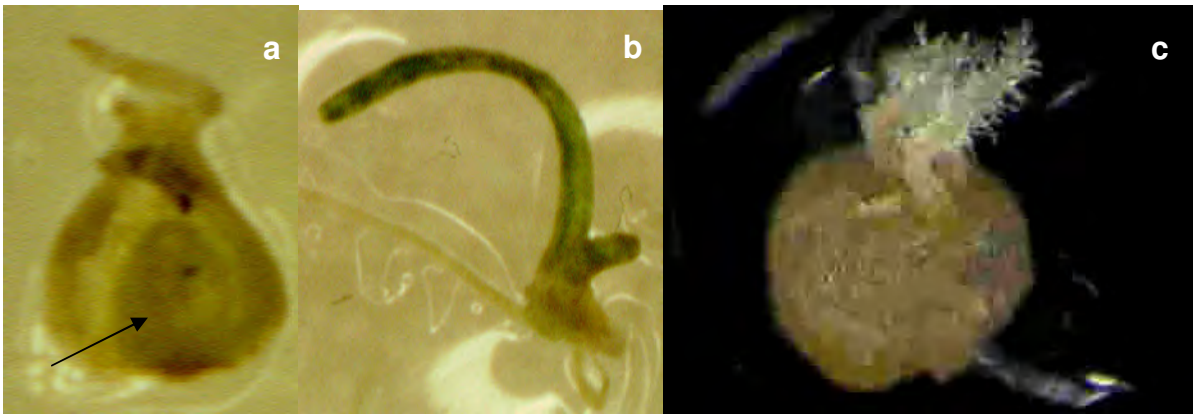

Fig. 25 a) Enlarged ovule (arrow) in the enlarged ovary **b)** germination of roots and shoots from the excised ovule of the enlarged ovary **c)** enlarged pistil that failed to germinate when isolated from the floret organs and cultured separately without the excision of ovules.

6.2.4 The growth of donor plant and harvesting stage of panicle: Both the physiological state of the donor plants and growing seasons influence gynogenesis in tef. Panicles taken in winter (January-March) were more responsive than that of summer grown plants (data not shown). Similarly, panicles harvested from the main tillers of vigorously grown plants at 2- 4 floret per spikelet (Fig. 18b) stage and between 14-17 cm long were superior and formed high ET formation compared with other stages for all varieties (Table 30). Moreover, as shown in Table 30 the growth stage and position of panicle at the time of culture highly affects gynogenic response since the stages of microspore are a major factor in androgenic responses of many species.

In tef when the basal two-three florets are matured or dehisced pollen, the upper consecutive florets of the same spikelet can respond to gynogenesis. In spikelet and panicle segment culture secondary floret growth was a common phenomenon. *In vivo*, the maximum number of florets per spikelet for variety DZ-01-196 is 10, but in this cultures a maximum of 35 florets (Fig. 24h) and 17 as an average per spikelet were observed. Hence, gynogenic florets are usually observed at the 12^{th} to 15^{th} floret numbers.

6.2.5 Effect of panicle part and 2, 4-D levels: The highest percentage of ET production were obtained from the intermediate part of panicle at all levels of 2, 4-D (Fig. 26) followed by the bottom and the top parts.

Table 30. Embryonic tissue (ET) formation and regeneration from various stages of panicle development of variety DZ-01-196.

Stage of panicle development (Panicle length, cm)	Number of spikelets cultured	Number of ET formed	% of ET	Number of regenerants	% of regenerants
0-5	210	0	0	0	0
6-10	430	19	4.40	6	1.3
11-13	660	42	6.36	21.	3.2
14-17	750	104	13.9	78	10.4
18-20	810	33	3.87	19	2.3

(Pooled data over two experiments)

Normally one or two basal florets of the spikelets on the top panicle position contain *in vivo* fertilised florets. These usually either continued zygotic development in culture and germinate to seedlings or rarely induce compact embryonic callus and regenerate plants in the same culture medium. Since these were very distinct from the ovaries forming ETs there was no confusion in data recording. However, avoiding the top positions is advisable. As Fig. 26 shows embryogenic ovaries and ETs production were increased by 69 % when the concentration of 2, 4-D was doubled and decreased dramatically. So in experiments to follow, 20 µM of 2, 4-D and the intermediate panicle parts were used.

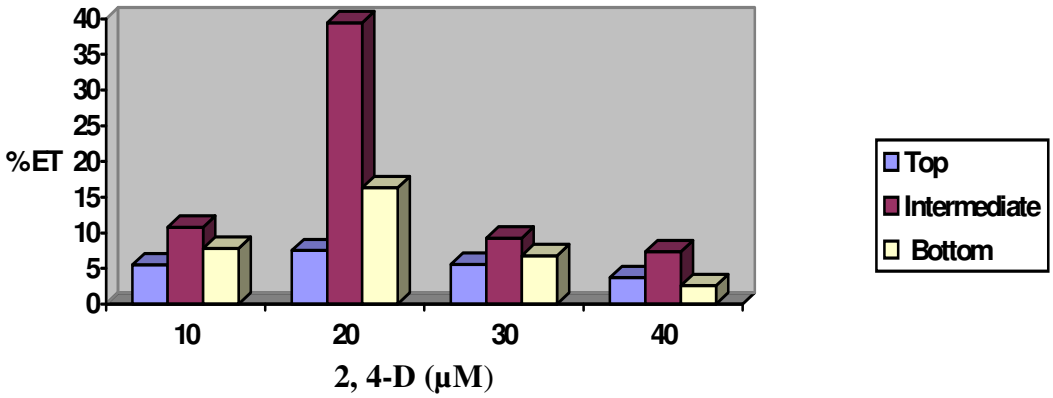

Fig. 26 Effe**ct** of 2, 4-D and panicle parts on ET Formation of variety DZ-01-196

6.2.6 Effect of panicle pre-treatment and explant type:

As indicated in (Fig. 27) there was high variation among the tested treatments on both panicle and spikelet cultures and pre-treatment of both explants. 36 h of pre-treatment gave the highest percent of ET formation followed by 12h and non treated control (fresh). Comparing the three types of explants the isolated pistils are totally unsuitable to *in vitro* gynogenesis where as spikelet culture gave low percent of ET formation compared to panicle segment culture.

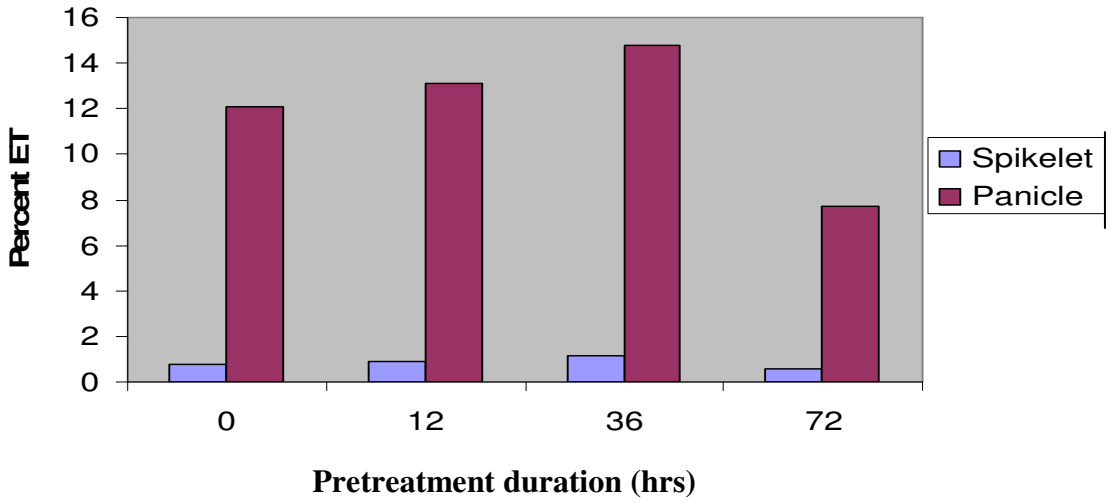

Fig. 27 Effect of pre-treatment duration on ET formation using spikelet and panicle segment culture of DZ-01-196

6.2.7 Response of genotypes: High variation was observed in the percent of ET formation and plant regeneration among the three tef varieties tested using 20 µM 2, 4-D (Table 31). Variety DZ-01-196 gave the highest percent of ET production (38.6 %) and plant regeneration (31.5 %) followed by Fesho (32.4 % & 25.5 %) and DZ-CR-37 (11 % & 10.2 %).

Table 31. Embryogenic tissue (ET) formation and plant regeneration from panicle segment culture of the tested genotypes.

Genotypes	Total number of spikelets	Number of ETs	% of ET formation	Number of regenerated ETs	% of regenerated ETs
DZ-01-196	1305	504	38.6	159	31.5
Fesho	1210	392	32.4	100	25.5
DZ-CR-37	800	88	11.0	9.0	10.2
E.mexicana	550	0	0.0	0	0.0

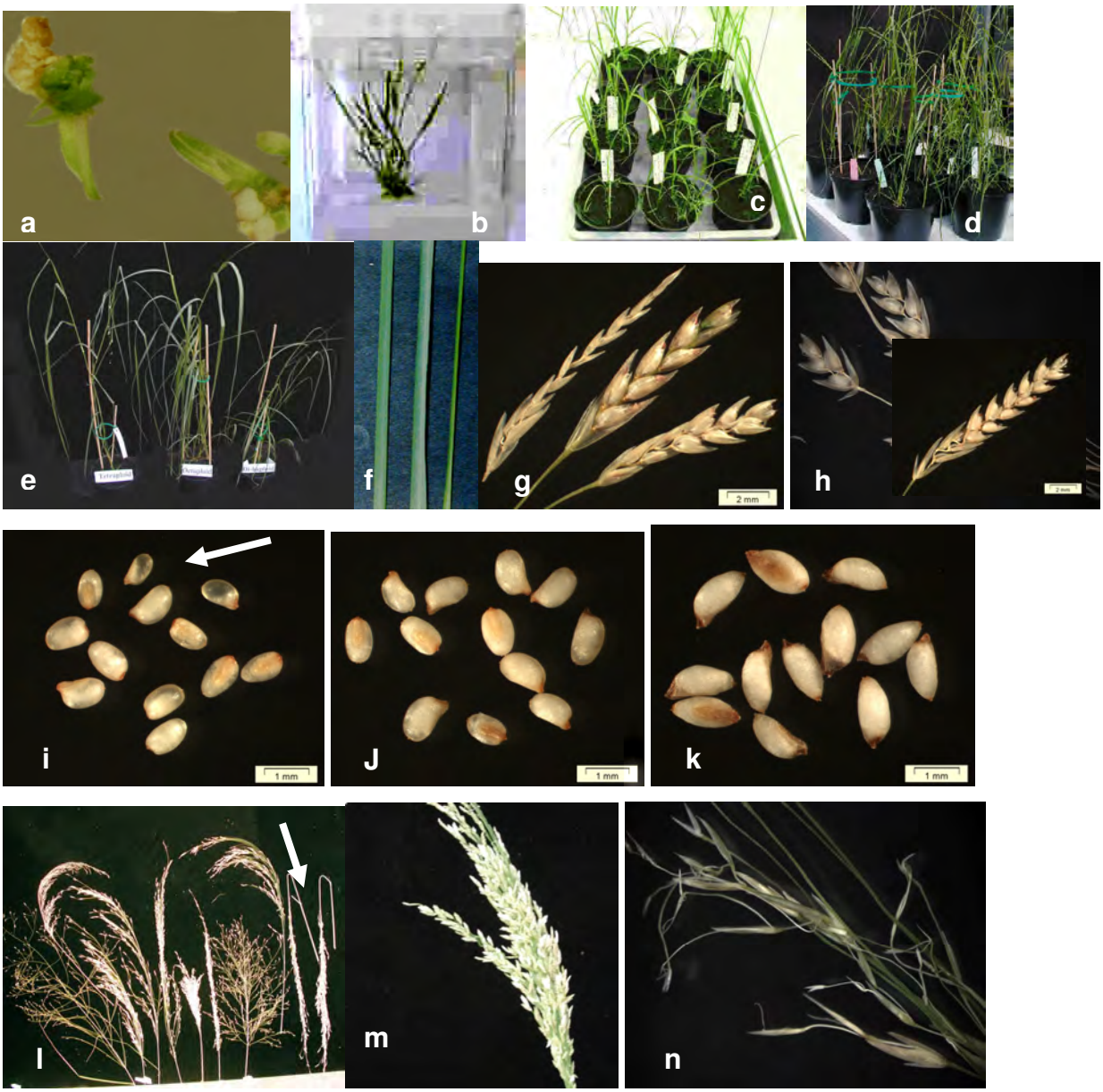

Fig. 28 Regenerants and various morphological variations including polyploids from the gyno derived tef variety, DZ-01-196 **a**) shoot regeneration from the enlarged pistils **b)** rooting in Magenta vessel **c)** regenerants in pots (no albinism) **d**) vigorous growth of regenerants **e)** the various polyploids produced haploid, octoploid and tetraploid from right to left **f)** leaf size variation of tetraploid, octoploid and haploid plants from left to right **g)** spikelet variation among the three ploidies **h)** variation in floret number per spikelet 17 and 4 florets per spikelet. **i, j** & **k)** seed size variation from left to right haploid with no endosperm (arrow), tetraploid and octoploid **l)** variation in panicle form from very loose to compact benders (arrow) **m)** spike like panicle **n)** panicle from aneuploid plants of variety DZ-CR-37 having deformed and underdeveloped inflorescences.

The low response of variety DZ-CR-37 could also be due to the abnormal panicle growth of this variety during winter. Repeated experiments were carried out within 5 months and from the total of 14723 spikelets on the panicle segments cultures of the three tested varieties of tef, 2045 (13.89 %) ETs were obtained.

6.2.8 Regeneration and plant growth: Prematurely detached ETs from the floret organs normally terminated growth, turned browny and failed to regenerate (Fig. 25c). However, matured ETs after 15-20 days of incubation were able to regenerate (Fig. 28a) when transferred to MSSI regeneration medium for 2- 4 weeks and transferred to Magenta vessel containing MS devoid of hormones (Fig. 28b).

A total of 182 plantlets were transferred to soil in the green house. Of these 26 % were regenerated to single plant per ovary and the rest ranged between 2-8 plants. There were no albino plants (Fig. 28c) among the regenerated plants. Almost all were vigorous showing morphological (gametoclonal) variations. These include variation in size of leaves, florets (Fig. 27 h) anther, pistil, stomata, seed and culm thickness; in length of flag leaf, plant height, and panicle length; in structure of panicle form and types of floret; in number of florets per spikelet; in maturity dates (early to late) and in fertility.

Distinct morphological variation was observed compared to the donor parent. The haploid plants had relatively thinner leaves, culms; floret and 99 % of them were sterile (Fig. 28g left). However, interestingly, one of the haploid plants with highly sterile panicles stored fertility after occasional branches appeared on the stem nodes of the secondary tillers after the main stem dies. These multiple, strong branches were relatively vigorous, strong with partial fertile panicle producing one to two seeds per spikelet. Most of the seeds had empty or partially filled endosperm and all are very small (Fig. 28i). Some of these seeds were potted and were germinated and produced fully fertile plants. The result of the flow cytometry analysis of these plants indicated twice as much chromosome as the di-haploids, i.e. tetraploids.

6.2.9 Flow cytometry: Flow cytometry is a simple and accurate method to measure nuclear DNA content in each cell and a frequency analysis of a large number of cells reveals peaks reflecting that amount within seconds. A total of 546 scans were run using the 182 regenerated lines compared with the seed lots controls of the same varieties. As speculated from the origin of ETs, 5 plants were haploids (di) haploids (2n=2x=20) (Fig. 29a), two were aneuploid from varieties Fesho and DZ-CR-37, 174 were tetraploid (2n=4x=40) and one was octoploid (2n=8x=80) (Fig. 29c).

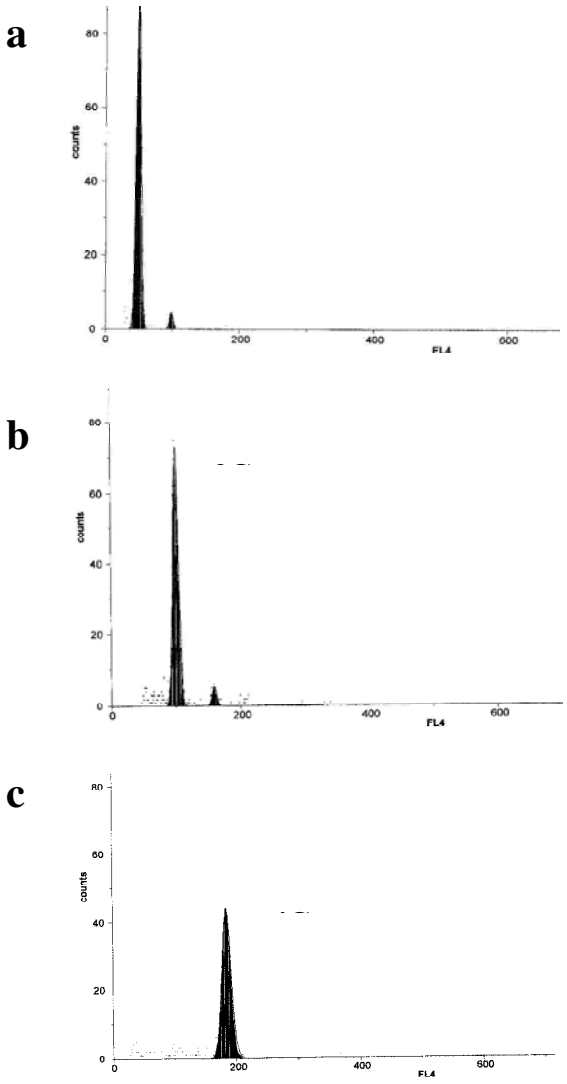

Fig. 29 a –c. Flow cytometry analysis histogram peaks indicating**: a)** 1C haploid nucleus DNA. **b)** control, tetraploid at **2C c)** octoploid plant at 4C

Peaks were evaluated by the coefficient of variation (CV) calculated on the computer. Since tef is an allotetrapoloid and its haploid has exactly half chromosome complement resulting two (di) haploid n chromosomes. The tetraploids having twice the DNA complement and number of chromosome than that of the diploid counterparts have peaks at 2C. So it was easy to detect the ploidy levels of the haploid plants since it shows peaks predominantly at 1C (Fig. 28a) and also the octoploid plant at 4C (Fig. 28c) indicating half of the genome nucleus at 1C and doubled at 4C respectively (Figs. 28 a-c). The aneuploid showed peaks at approximately 1.5C (data not shown).

The aneuploid plants developed very abnormal panicle branches and spikelets mostly with under developed florets (Fig. 28n) and did not set seed at all. The octoploid plant rather has very thick and wider deep green leaves, short height, partially fertile and delayed maturity.

Generally most of the regenerated plants were vigorous in morphology but inferior in fertility. There were no apparent ploidy levels on the measured control plants of the tested varieties. All showed tetraploid levels.

In general, evaluation of the 182 gynogenic plants for gametoclonal variation such as; fertility, number of stomata, leaf width, the various panicle types other than the fairly loose panicle of DZ-01-196 and yield revealed high gametoclonal variations. Panicle forms such as spike type, very loose panicle and bending types at the peduncle were not present in the parent genotype, DZ-01-196. Besides, few plants were obtained with vigorous growth and heavy panicle weight but with poor seed setting and high sterility.

6.3 Discussion

Several factors may influence the success of gynogenesis in tef and some are discussed in detail below.

6.3.1 Genotype and donor plant growth condition: From the four genotypes tested in the panicle segment culture, the response of variety DZ-01-196 was the highest followed by varieties Fesho and DZ-CR-37. The wild species, *E.mexicana* did not respond for this culture condition,

however, showed high response for excised pistil culture. This might be due to the genotypic response variation or due to the growth condition of parental plants. Panicles harvested from variety DZ-CR-37 were usually abnormal in flower commencement and development under abnormal growth condition (16 or 8h light). Besides, explants collected from the un-healthy and old plants were low in gynogenic response. Panicles harvested from the main tillers of the normally grown plants were more responsive.

6.3.2 Stage of ovary growth: Stage of ovary development is very critical in *in vitro* gynogenesis. For instance in barley gynogenesis the largest number of haploid plants were produced from ovaries at the tri-nucleate stage (Castillo and Cistue, 1993). In rice ovary culture (Zhou and Yang, 1981, Zhou et al., 1983) critical inductive period takes place during megagametogenesis instead of megasporogenesis which mostly ranged from uninucleate to four-nucleate embryo sacs stage corresponding to late uninucleate to early bi-nucleate pollen developmental stage. According to these authors further matured ovaries were not highly responsive. However, in our experiment variation was exhibited among the two *Eragrostis* species and the excised unpollinated pistil culture; tef pistils showed no response when the ovaries were matured but, *E mexicana*'s ovaries responded well at this stage. In onion the embryogenic ovaries were at 7 nuclei stage when ovules induced embryonic tissues (Ge´me´s-Juhasz et. al., 2002).

Mengesha (1966) studied the embryo sacs of tef and revealed the absence of apomixis and pistils at the stage of self pollination can have 16-19 antipodal cells. These matured stage of tef ovaries probably at or before self pollination were too late to switch to parthenogenic development initiated by external stimuli in this culture condition.

According to the review by Zhou et al., (1986) microscopic observation of rice ovaries revealed that gynogenesis did not trigger until the cultured immature ovaries reached gametophyte stage. This observation was in agreement with tef gynogenic studies since the small ovaries were grown in culture medium until it reached to the stage where gynogenesis can be initiated.

In tef it is possible to find young explants in the intermediate to-top part of panicle while the bottom two-three florets were self pollinated and gets older. Since it was not easy to observe the embryo sacs developmental stage by simple microscopic observation, microspore growth stage

can be used since it is feasible to detect the ovary growth without sectioning for microscopic observation similar to that of rice (Zhou et al., 1986).

6.3.3 Explant type and floral parts: The other interesting observation in tef was, in contrast to some cereals such as barley (Wilson, 1977) and sorghum (Brettell et al., 1980), where the whole panicle and inflorescence culture respectively, are used as an aid for androgenic microspore development, resulting only to gynogenic response while the anthers degenerated and failed to undergo meiosis to form the haploid microspores.

Immature spikelets and panicle segments were the only responsive explant types in tef. The unsuccessful result of the isolated pistil culture of tef could be related with the reports found in both androgenic and gynogenic haplodization of many crops such as onion and mulberry. For instance, Campion and Allon (1990) in onion and Thomas et al., (1999) in mulberry gynogenesis suggested that the effect of cultures is at least partially mediated through the levels of endogenous substances within the spikes or florets. Moreover, many experiments on pollinated ovary culture of rice (Maheshwari and Rangaswamy, 1965) have shown that floret parts such as pedicel, calyx or glumes played an important role in fruit growth and embryo development. Similarly in rice gynogenic study using ovary culture, the best result was obtained when an unhusked flower with intact pistils, stamens and glumes attached to a piece of receptacle was cultured as a unit while single pistils detached from receptacle however, did not respond (Zhou and Yang, 1981a; Zhou et al., 1983). These results were in agreement with panicle/spikelet culture of tef compared to the excised pistil culture. In barley also the whole flower, with or without stamen was proved to be a better explant than randomly oriented single pistil (Huang et al., 1982). Therefore, the supportive effect of floral parts including the stamens in cereals seemed to be attributed to the supply of nutritive and active substances contained in them for gynogenesis.

On the other hand, the induced calli of tef from the excised pistil culture did not regenerate to plants. Similarly Thomas et al., (1999) reported that individual ovaries growth of mulberry was very poor in culture and gynogenic plants were never formed out of the induced calli. In contrast, in the culture of un-pollinated isolated ovaries of barley (Castillo and Cistue, 1993) and rice (Kuo, 1982), calli were induced and regenerated to shoots and roots but, in tef only roots were

regenerated from the cultured isolated pistils. However, in using panicle segment culture of tef, there was no callusing phase for inducing the ETs. Therefore, the floral parts seem very essential for plantlet regeneration because, the excised embryogenic ovaries which formed ETs terminated growth and did not regenerate when the floral parts were removed.

The degeneration of the anthers, the formation of (white spongy mass tissues) and the sprouting of embryo from the culture of unpollinated pistils of tef in panicle culture, without the callus phase seemed to be an evidence for the development of non zygotic and or somatic embryogenesis. Hence, it can be speculated that mainly the egg apparatus or most of the embryo sac cells (the synergies, central cell and antipodals) might be triggered to mitotic division by the external stimuli applied *in vitro* which is correlated with light stimulated repeated division of the female gametophyte. Identifying the origin of the gynogenic embryogenesis from the embryo sac cells of tef would be very important for microscopical ontogenetic studies.

6.3 4 Effect of exogenous hormones: The embryo sac of tef was triggered by the high level of exogenous hormones to initiate parthenogenic development. This response was not observed in both panicle segments and isolated pistils cultured at low level or devoid of exogenous hormones (data not shown). Addition of high level hormones 2, 4-D (10-20 μM) was the most effective factor in tef gynogenesis. Omission of the hormones inhibited gynogenic response. In rice ovary culture, low concentration, 0.125-0.5 mg/L of 2-methyle-4-chlorophenol (MCPA), an auxin, was used routinely (Zhou and Yang, 1981). Higher concentration, 2 mg/L or above of MCPA normally decreased gynogenic induction of rice ovaries, but facilitated somatic embryo induction from the ovary wall or receptacle. It is also known that the enlargement of ovaries after natural fertilization is related with the raising of the endogenous auxin (IAA) level by the sperm cells in to the embryo sac. Berhe and Miller (1976) stated that the embryo sac of tef grew due to the applied hormones from the fertilizing male gametes.

6.3.5 Pre-treatment (cold): In contrast with anther or microspore culture, gynogenic approach in tef did not show the need of cold pre-treatment. However, induction frequency increased by the cold (4°C) storage of panicle prior to inoculation for 36 hours. Bohanec and Ja'kse (1999) also stated that unlike androgenesis, *in vitro* gynogenesis is generally not stimulated by a shock treatment or pre growth on starvation medium. However, certain physical treatments (e.g., Low

or high temperature) applied to the donor plants or excised explants may have a strong influence on embryo induction. As a result, high embryo induction frequency of 18.4 % was obtained when cucumber ovary slices were pre-incubated at 35°C in the dark (Ge´mes-Juha'sz-et al., (2002). Similarly Puddephat et al., (1999) obtained a ten-fold increase in embryogenesis when reconditioning of stock plants maintained at 15°C compared to 10°C. In tef both treated and untreated explants in cold (4°C) or in hot (32°C) for a short period have shown pistil enlargement although, gynogenic response efficiency increased with cold pre-treatment for short duration. This highly distinct variation was not observed in cold (8-10°C) treatment of rice panicle for gynogenic response (Zhou et al., 1986).

6.3.6 Culture medium and light: For pistil enlargement, the presence or absence of light has no impact. However, for embryogenic tissue (ET) formation, the effect of light had much influence in all cultured genotypes. There were no ETs formed from the cultured panicles segment at dark however, the panicle segments and spikelets cultured at dark showed elongation of the rachis and increased floret number without showing gynogenesis and rather pistils were enlarged and collapsed after few days. Therefore, culturing under light is very important factor for tef gynogenesis; however, little work has been done to study the effects of light on embryonic development. In tef gynogenic culture the reason why light (16/8 h day/night) was correlated with mild temperature (22 ±2°C) is not clear.

6.3.7 Polyploidy level and morphological variation of regenerated plants: The flow cytometry result clearly indicated small proportion of mixiploidy (various levels of ploidy) in gynogenic regenerants of tef. Polyploidy level other than tetraploid had never been reported for tef. Ayele et al., 1996 studied tef genotypes for a possible existence of variant polyploidy in tef using 35 local cultivars of Ebba (1975) and some improved varieties including DZ-01-196, but they reported the absence of mixiploidy in tef. The presence of various polyploidy in this culture condition is due to gametoclonal variation initiated from the gynogenic tissues of tef.

In gynogenesis studies of many crops generally, there is a greater haploid portion and smaller diploid portions compared to androgenesis. On the other hand, since embryological observations have precluded doubts of the somatic origin, the diploid were thought to be due to chromosomal

doubling events during the course of gynogenesis (Zhou et al., 1986). This could be the case for the tetraploid and octoploid origins in tef gynogenesis.

The rarely induced calli from the somatic cells of zygotic embryos of fertilized ovaries were easily distinguished at early observation because ETs were morphologically distinct from the somatic ones. The other main distinction between the two cell origins was that most of the ETs were sprouted from the ovary wall below the white spongy mass so the ETs could be derived only from the gynogenic origin. This is in agreement to Zhou et al., (1986) who stated almost all masses dissected from inside of the ovaries were of gynogenic origin.

These ample gametoclonal variations including the aneuploids increase the diversity of the crop and those traits could be included in the future breeding program. According to Singh (1986), aneuploids occur spontaneously and may be produced in high frequencies from triploids, auto tetraploids, tetrasomic plants and translocation heterozygotes. The occurrence of variation in the gynogenic regenerants especially the tetraploids could be due to the spontaneous doubling that may have occurred *in vitro* from the allotetraploid tef origin. Therefore, it is speculated that the majority of the tetraploid plants could originate either from the *in vitro* spontaneous doubling of the haploid cells of the embryo sac or from the unfertilized polar nuclei which are 2n in their chromosome complement.

6.3.8 Seed-setting of the haploid plant: Reports of seed set on haploid plants are very rare but, Jauhar et al., (2000) reported this phenomenon, in self pollinated wheat haploids that were obtained from the crossing with maize pollen (wide hybridization). Here viable seeds were formed in all genotypes although the seed set percent varied between the genotypes. Those seeds gave rise to normal disomic (2n=4x=28 AABB) plants. Similarly seeds were obtained from the haploid tef plant which has viable embryos and germinated to normal tetraploid plants. The flow cytometry result of the second generation of both the haploid and octoploid plants revealed that tetraploids and octoploid plants were obtained respectively.

According to Jauhar et al., (2000) the viable embryos of the haploids of wheat might be formed by fusion of the unreduced male and female gametes. That is the unreduced gametes of wheat were formed by two closely related first division restitution mechanisms resulting in meiotic

non-reduction (i) complete failure of movement of univalent at anaphase I, followed by normal second (equational) division and (ii) anaphase I movement of all univalent to one pole. Some seeds of the tef haploid plant which were grown in the green house produced fully fertile progenies with twice as much chromosome as the parent (tetraploid plant).

In many other distant hybrids, occasional unreduced gametes are produced thus giving rise to allopolyploid progeny. The allotetraploid *Raphano brassica* was obtained in this manner, the F_1 from the cross *B. oleracea* (cabbage) x *Raphanus sativas* (radish) was almost completely sterile, but produced a few seeds that gave rise to the allotetraploid *Rophano brassica* (Singh, 1986). This could be the case for tef seed set from the haploid plants.

The present work is the first successful report of *in vitro* gynogenesis for the production of haploids in *Eragrostis tef* using panicle segment culture. Besides, this gynogenic research work is the first novel finding for developing new genotypes with various levels of polyploidy. Hence, gynogenesis can be used as a main tool for the haploid production in tef since androgenic approach may be recalcitrant or inapplicable to some species and genotypes and in case it is applicable, the production of albino plants that is high in the latter techniques may be avoided through gynogenesis.

This protocol developed for tef gynogenesis for the production of haploids and presumably double haploids (DH) will shorten the breeding cycle of tef from many to few years and can increase selection efficiency. So the release of new varieties with desired agronomic traits could become easier for various agro ecologies in the near future.

6.4 References

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Chapter 7

Tef Transformation

7.1 Materials and Methods

7.1.1 Plant material and tissue culture: Callus was initiated from immature embryos of tef variety, DZ-01-196 as described in chapter 3. The transformation experiments were performed at the IPK Gatersleben, Germany.

7.1.2 Transformation vectors**: The transformation vectors used were comprehensively described in Table 32. The plasmids were kindly provided by Dr. Silvia Broeders (pSB212) and Dr. Goetz Hensel (pYF132, pYF133) (IPK Gatersleben, Germany). The binary vectors pSB212 and pYF133 were also provided in the *Agrobacterium tumefacies* strain AGL1 (Lazo et al., 1991).**

7.1.3 Embryo sensitivity test: The sensitivity of proliferating immature tef embryos towards the selective agents to be used was pre-determined. Various levels of bialaphos (10, 20, 30, 40, 50 and 60 mg/l) and hygromycin (2.5, 5.0, 7.5 and 12.5 µM) were tested (Table 34). For paramomycin, the level suitable for wheat (25 mg/l) was found sufficient at the first attempt to kill all none-transformed control embryonic calli in tef.

7.1.4 Direct DNA transfer using the biolistic method: Biolistic transformation of tef was carried out using the particle acceleration device PDS1000/He (BioRad) as described by Altpeter et al., (1996). Thirty mg gold particles of 0.6 µm and the same amount of 1.0 µm particles were mixed, suspended in 1 ml of 70 % ethanol and vortexed for 3 minutes followed by 10 seconds of centrifugation at 14000 rpm. The supernatant was removed and the procedure was repeated once. Then, the pellet was resuspended again in 1 ml of 70 % ethanol. Thirty µl of the resultant suspension were transferred into a 0.75 ml sterile Eppendorf tube and centrifuged at 14000 rpm for 10 seconds. The supernatant was removed and the pellet resuspended in 50 µl of sterile doubled-distilled water for washing. The gold particles were pelleted by centrifugation as before

and resuspended again in 55 µl of sterile doubled-distilled water. Five µl of plasmid DNA (1µg/µl stock) was added followed by intense vortexing for 1 minute. Then, 20 µl of 0.1 M Spermidine and 50 µl of 0.25 M $CaCl_2$ were added for precipitation of the DNA onto the gold particles. After having mixed the suspension on a vortex at medium speed for 3 minutes the particles were pelleted by centrifugation as before. The resultant pellet was washed with 200 µl of 100 % Ethanol followed by centrifugation, the supernatant removed and the pellet resuspended finally in 210 µl of pure Ethanol. Immediately prior to loading the suspended particles onto macroprojectiles, the Eppendorf tube was sonicated briefly. For each bombardment 3 µl of the suspended DNA-coated particles were loaded per macroprojectile. Osmoticum medium was prepared by supplementing K99SE with 0.65 M mannitol which is a metabolically inert sugar alcohol used in tissue culture to provide appropriate osmotic conditions without addition of excess nutritional carbon source. About 20-40 embryos, were arranged in a compact cluster in the center of a 6-cm petri dish containing solidified osmoticum medium for 4-6 hours before bombardment. Each plate was bombarded once by the particle gun with a rupture disc providing 1100 psi and the distance between the stopping mesh and explants adjusted to 5 cm. Following bombardment, petri dishes were incubated at 25±1°C in the dark for 18 h and were then transferred to the normal somatic embryo induction medium (K99SE, see Chapter 5).

7.1.5 Agrobacterium transformation: **Plant materials and culture initiation was conducted as described for the biolistic method, except embryos were not transferred to osmoticum medium. About 60 embryos per treatment comprising three independent replications were used. *Agrobacterium* strain AGL1 containing either pSB212 or pYF133 (Table 32) was co-cultured with immature tef embryos either freshly isolated or precultured for 2-5, 6-7 or 8-14 days in co-culture dishes containing 2.5 ml of liquid CCM medium as described by Hensel and Kumlehn (2004). After co-culture, the wells were maintained in the dark at 20°C for 48-72 h. The established cultures were transferred to tef embryo culture medium (K99SE) containing 200 mg/l Timentin to avoid further bacterial growth and incubated at 26° C in the dark.**

7.1.6 Selection of transgenic plants: Only explants which had formed embryonic callus were selected and transferred to the respective selection medium (same medium containing selective agent according to (Table 32) after various days of resting periods (see Table 33). Shooted calli were sub-cultured onto fresh selective media of the same composition at 14 d intervals. The regenerated plants were compared with both positive (non-transformed material cultured without

selective agent) and negative (non-transformed material cultured on media containing the respective selective agent) controls. Green shoots were transferred to glass tubes containing the same medium. After two weeks plantlets which survived selection were transferred to soil.

7.1.7 Analysis of transient Gfp expression: **To analyse transient Gfp expression, bombarded scutellar tissues were examined 2 days after transformation under a microscope equipped with UV light and an appropriate emission/excitation wave length filter set.**

7.1.8 Molecular analyses**: Leaf samples from both treated and control plants were taken for DNA extraction and stored at -20°C. Total genomic DNA was isolated from the leaf samples as described by Palotta et al., (2000). Candidate primary transgenic (T_0) plants were checked by PCR using appropriate primer pairs (Hensel and Kumlehn, 2004) for the respective sequences transferred. For Southern hybridisation, 15-20 µg aliquots of genomic DNA of PCR-positive T_0 plants were digested at 37°C for 5-6 h with enzymes that have unique restriction sites in the corresponding DNA sequences transferred. The DNA was size-fractioned by gel electrophoresis using 0.8 % (w/v) agarose and capillary-blotted as essentially described by Sambrook et al., (1989) onto a Hybond N^+ membrane under alkaline conditions as described by the manufacturers' instructions (Amersham, Braunschweig, Germany). The membrane was hybridised with an Hpt probe labelled by DIG-dUTP using PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany), the primers 5'-GATCGGACGATTGCGTCGCA-3' and 5'-TATCGGCACTTTGCATCGGC-3' as well as plasmid pYF133 as template DNA. Hybridisation and DIG detection using CDP-Star was performed as described in the 'DIG Application Guide for Filter Hybridization' (Roche Diagnostics, Mannheim, Germany). The signals were visualized using Chemiluminescent Detection Film (Roche Diagnostics, Mannheim, Germany).**

Table 32. Description of vectors used for both biolistic and *Agrobacterium* transformation of tef

Parameters	**Particle bombardment**		**Agrobacterium**	
Transformation vector	pYF 132	pYF133	pSB212	pYF133
Reporter gene cassette	MaizeUbiquitin promoter::*Gus*	MaizeUbiquitin promoter::*Gfp*	RiceActin1 promoter::*Gfp*	MaizeUbiquitin promoter::*Gfp*
Selectable	CaMV35S	CaMV35S	CaMV35S	CaMV35S

marker gene cassette	promoter::*NptII*	promoter::*Hpt*	promoter::*Bar*	promoter::*Hpt*
Vector backbone	pCambia1300	pCambia1300	pLH7000	pCambia1300
Selective agent concentration used	Paramomycin, Sulphate, 100 mg/l	Hygromycin, 5 mg/l	Bialaphos, 50 mg/l	Hygromycin, 5 mg/l

7.1.9 Optimizing resting and agro-stop periods for both methods: Several durations of resting and agro-stop periods (0, 3, 7 and 14 d) (Table 33) for biolistic and *Agrobacterium* transformation methods, were investigated to avoid premature death of the transformed cells by immediate transfer of the treated tissues to the selection media.

7.2 Results

7.2.1 Reporter gene expression: *Gfp* expression was detected in explants two (Figs. 30a and b) and 7 days (Fig. 30c) following biolistic as well as *Agrobacterium* transformation. The number of GFP signals expressed in bombarded tissue was higher than following *Agrobacterium* transformation (Figs. 30b and c). When expressing tissues were transferred to selection medium and light after a total of 21 days of culture in the dark, most of them formed shoots within the first culture week. Yet, after sub-culture, the majority of shoots died and only few resistant plantlets developed (Fig. 31c).

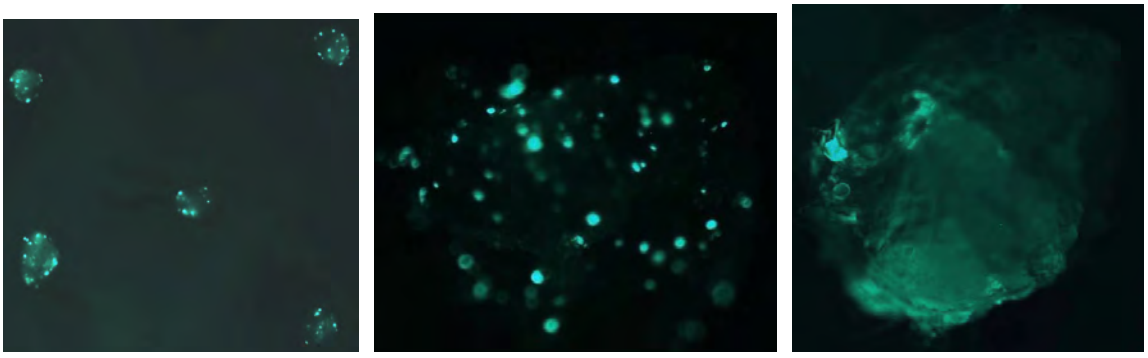

Fig. 30 GFP expression from the treated tissues of tef **a)** bombarded immature embryos two days after treatment **b)** a close look to transformed cells showing GFP expression in bombarded tissue **c)** expression of GFP from *Agrobacterium* treated tissue 7 days after co-culture.

7.2.2 Sensitivity test:

The preliminary observation using G418 indicates that immature embryos were not hindered from callus induction and somatic embryo formation on medium containing 50 mg/l G418. There was no inhibition of callus growth and formation of somatic embryos at 2-8 mg/l bialaphos in the medium. By contrast, the cultured explants showed high sensitivity towards hygromycin. Therefore, 10-60 mg/l and 2.5-12.5 µM were tested for the two selecting agents, respectively. There was a linear decrease of somatic embryo formation with increasing rates of both chemicals. 50 mg/l of bialaphos and 5 µM hygromycin completely inhibited somatic embryo formation and regeneration (Table 34). At higher levels of hygromycin even complete inhibition of callus formation was observed. Even at the optimum levels calli showed retarded growth and turned brown showing susceptibility. However, plants regenerated at a low rate and formed new shoots at these levels. On the other hand, the level of paromomycin (25 mg/l) was sufficient to kill all non-transformed control explants as it is also standardized for wheat transformation (J. Kumlehn, personal communication).

Table 33. Percent of regenerated plants after various days of resting and agro stop periods of tef using biolistic and *agrobacterium* transformation methods: 50 mg/l bialaphos containing RM.

Treatments **Biolistic transforma** ***Agrobacterium*** **metho**

Treatment days	Sub-culture	Resting period (days)				Agro-stop period (d)				untreated
		0	**3**	**7**	**14**	**0**	**3**	**7**	**14**	control
Total explants		60	60	60	60	60	60	60	60	60
No of SE formed		28	39	43	57	0	12	31	49	60
No of regenerants	-	10	26	38	46	0	5	22	45	58

Total No of regenerants	1st	10	16	33	43	0	2	17	35	58
Total No of regenerants	2nd	8	11	26	39	0	1	13	29	58
% of survived regenerants after 2nd subculture)		13.3	18.3	43.3	65.0	0	1.7	21.7	48.3	96.7

7.2.3 Resting and agro stop periods

Following *Agrobacterium* transformation, none of the treated embryos showed somatic embryo development when directly plated on a medium containing selective agent, even for the older explants (pre-cultured for14 days). This could be due to the presence of the selective agents (paramomycin, haygromycin and bialaphos) which might have killed even the transformed cells at a young stage.

Table 34. The level of resistance of fresh immature embryos for bialaphos and hygromycin levels.

Data measured	**Bialaphos (mg/l)**						**Hygromycin (µl)**			
Treatment levels	**10**	**20**	**30**	**40**	**50**	**60**	**2.5**	**5.0**	**7.5**	**12.5**
Total explant	60	60	60	60	60	60	60	60	60	60
No. of SE formed	57	49	36	17	2	0	35	5	2	0
Total No. of regenerants	56	44	31	12	1	0	17	3	2	0
1st	56	43	31	10	1	0	15	2	0	0
2nd subculture	55	42	29	10	1	0	15	1	0	0
% of regenerants after 2nd subculture	91.7	70.0	48.3	16.7	1.7	0	25.0	1.7	0	0

Therefore, efficient resting and agro stop periods (0-14 days), prior to transfer of bombarded as well as of agro co-cultured explants to the selective medium, was established to avoid escapes and premature death of the transgenic cells. Regenerated plants were obtained without a resting period after biolistic transformation. Similarly, 3 days resting period prior to agro stop gave only 1.7 % regeneration compared to 21.7 % after 7 days and 48.3 % after 14 days of resting (Table 33).

Therefore, no resting period for bombardment and 3 days for agro stop were found to be suitable for somatic embryo formation and transformed plant regeneration.

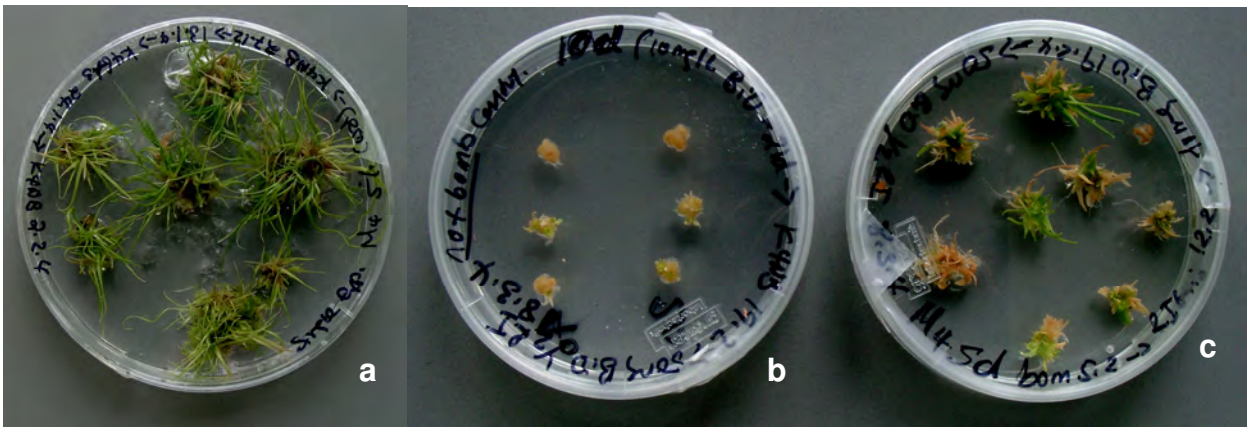

Fig. 31 a) Positive control (non-bombarded materials) cultured on media without selective agent, **b)** negative control (non-bombarded material cultured on media containing the selecting agent) **c)** regeneration of bombarded materials in the presence of selective agent

7.2.4 Molecular analyses and transgene expression:

Transformed genes were detected in analysed leaf tissues following both transformation methods using PCR and Southern blot analyses (Fig. 32 and Fig. 33). Four and one transgenic plants were obtained via biolisitic and *agrobacterium* transformation respectively. Presence, stable integration and generative transmission of the transgenes were unambiguously shown by these molecular analyses. Following biolistic transformation, all four transgenic plants obtained carried multiple copies of the transgenes. In turn, the plant obtained by *agrobacterium* transformation carried a single copy of the transgene, as is shown by Southern blot (Fig. 33).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

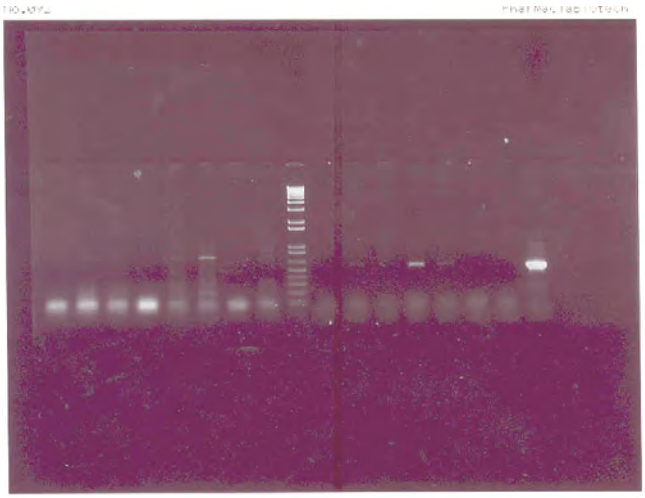

Fig. 32 PCR products in agarose gel electrophoresis showing transgenics, T_0 (lane 6 for GUS gene and lane 13 for GFP gene) and control (lane 9 and 17, respectively) developed through biolistic technique.

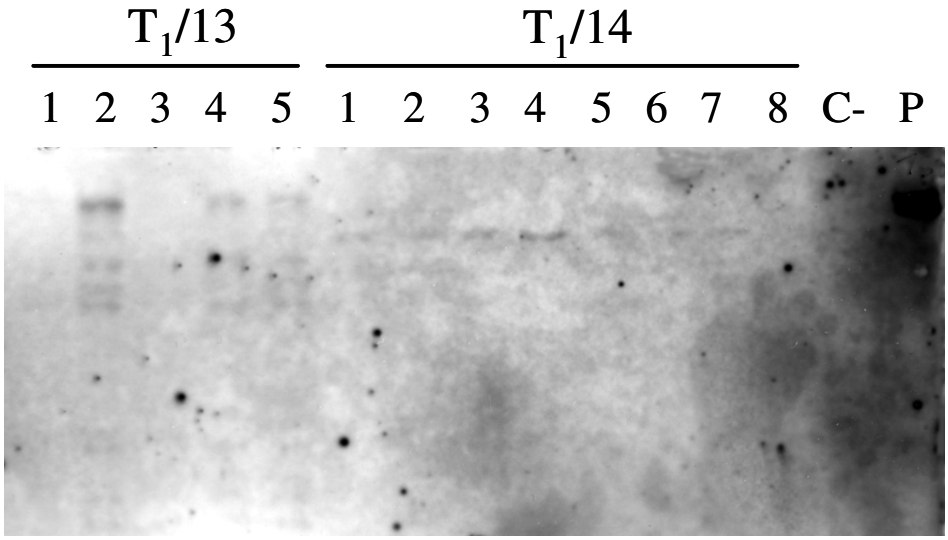

Fig. 33 Southern blot showing HindIII-digested genomic DNA of 5 and 8 progeny plants from two primary transgenic tef plants (plant 13 from biolisitic transformation, plant 14 from *agrobacterium* transformation), respectively, hybridised with a *Gfp* gene-specific DIG-labelled probe. Note that the multiple transgene copies derived from plant 13 are likely to be coupled on one and the same chromosome, since there is no segregation of the bands within its progeny, and a single-copy transgenic plant was obtained using *agrobacterium* transformation as is reflected by only one band per lane in all transgenic progeny plants. Plants 13-1, 13-3 and 14-8 are non-transgenic segregants.

Anthers of T_1 plants obtained via agrobacterium transformation using AGL1 (pYF133) showed 1:1 segregation of GFP expression (Fig. 34), as is expected for single-copy integration of the transgene.

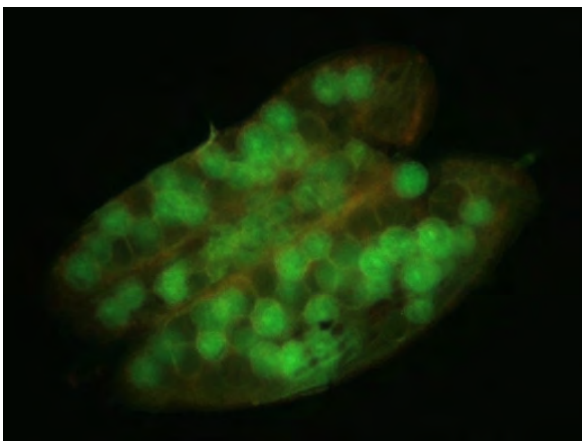

Fig. 34 Anthers of T_1 plants obtained via Agrobacterium transformation using AGL1 (pYF133) showing a 1:1 segregation of GFP expression

7.3 Discussion

Suitable selection markers are needed to give high levels of resistance in the transformed cells so that transformed cells survive while the wild type growth is inhibited by the addition of the appropriate dose of the selective agent in the media.

In this study, high level of (50 mg/l) bialaphos resistance was observed for callus and somatic embryo formation for the immature embryos of tef compared to hygromycin (5 µM). Tef tissues are highly susceptible for this chemical compared to bialaphos. This suggests that hygromycin could be an efficient selection agent for tef immature embryos. Transgenic plants were obtained in this study using both selection systems, provided appropriate concentrations were used. Mengiste (1991) showed the appropriateness of kanamycin resistance for callus tissues and suspension cultures of tef. However, tef tissues are very resistant to high levels of kanamycin and G418, although the latter at a very low level. Besides, natural resistance to kanamycin has been seen in some gramineae monocots (Christou et al., 1988). Similarly carbenicillin at a concentration commonly used (500 mg/l) for arresting the growth of bacteria at the end of the co-cultivation period has been reported to increase callus growth similar to hormones instead of inhibition. Therefore, Mengiste (1991) recommended not using this chemical in tef *agrobacterium* transformation.

In many transformation works the need for wounding site for the purpose of transformation was required and in tef and some horticultural crops also the importance of this site is reported, although attachment of the *agrobacterium* was shown in the non-wounding sites also (Mekbib et al., 2001). This observation is similar to the result of this study since the freshly isolated immature embryos without forming calli were able to accept the *agrobacterium* infection in co-culture condition only.

The result of this protocol is the first in kind to produce transgenic tef lines which in turn will facilitate the improvement of the crop in the near future since lodging resistance genes may be transformed from other cereals crops like wheat of Rht dwarfing gene.

7.4 References

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Chapter 8

Summary and recommendation

8. 1 Embryo/ovary rescue

Three tef varieties, DZ-01-196, DZ-CR-37 and Fesho and three wild and close relatives, *E. pilosa*, *E. mexicana* and *E. papposa* were used for embryo rescue experiments. Both zygotic embryos and ovaries at early stage of self pollination were excised aseptically and cultured in K99 medium. The effect of immature explants age, media composition, glutamine levels, culture days in the dark (incubation period), optimal explant types and responses of genotypes were studied and generated several useful results. The highest plant regeneration was obtained from variety DZ-01-196 embryo and ovary at the same percentage (98 %) followed by DZ-CR-37 (95 % and 75 %) and Fesho (78 % and 75 %) for ovary and embryo, respectively. More than 90 % rescue was also recorded for all wild species from embryo culture. K99 medium was optimized for the high rescue of *Eragrostis* species by supplementing with 250 mM maltose and devoid of both glutamine and 2, 4-D. The required dark culture for ovaries was only 5-7 days whereas; immature embryos required 14-30 days depending on the age of explants. Based on the data obtained, the protocol for embryo rescue technique for *Eragrostis* species is successfully drawn and it is likely that inter-specific cross hybrids can be achieved in the near future for tef improvement research.

Therefore, the tef breeding research group should use this protocol to further rescue of interspecific crosses, which were either aborted at early stage or loose viability. Although the established technique is very useful still the need for refinement of the technique for further rescuing the zygotic embryos and ovaries at their extreme immature stages such as 1-2 days after pollination for ovaries and 1-5 days for embryos need further work.

8.2 Somatic embryogenesis

A highly efficient *in vitro* culture system was developed from immature embryos of tef and it depends on (1) the developmental stage of embryos, (2) type of genotypes used (3) various levels of glutamine, 2, 4-D, maltose and phytagel in K99 medium during callus induction phase. The formation of somatic embryos and regenerable callus of three tef varieties namely DZ-01-196,

DZ-CR-37 and Fesho were compared on the mineral compositions of MS and K99 media. 100 % somatic embryo formation and plant regeneration (100 %) as well as a maximum of 565 plantlets per single explant were obtained from variety DZ-01-196 using an intermediate size embryos cultured on K99 optimized medium and transferred to K4NB regeneration medium. Somatic embryos were formed within 14 days and the embryogenic calli transferred to the regeneration medium highly proliferated (induced) secondary somatic embryos which substantially increased the number of plantlets as splitting and sub-culturing continued to 8-10 weeks at 14 days interval. There was also a highly significant correlation between explant size and genotype and the tested variants for somatic embryo induction and regeneration. Fesho was the least responsive variety and it requires further study to optimize its regeneration medium. Glutamine and phytagel were the most prominent factors for immature embryo cultures in tef. The growth and fertility of the plantlets grown in the greenhouse were without any morphological and fertility disorders. This protocol provides a highly applicable and fast plant regeneration system, which is necessary for future application.

It is recommended that this protocol be used for three purposes i) cloning or multiplication of the rarely obtained seeds or F_1 hybrids ii) as an alternative tool for embryo rescue by excising and culturing immature embryos at their young stage, iii) *in vitro* selection for various traits like resistance to diseases, salt tolerant and drought, iv) transformation studies. In general, using the intermediate size of embryos is more convenient to excision since they are more productive than the other stages.

8.3 Androgenesis study in tef: Anther and Microspore culture

In this study efficient microspore isolation and purification techniques, the effect of various pre-treatment including starvation and temperature shock, co-culturing with ovaries and a week old dividing ms of barely, for the purpose of feeding and initiating stimuli and responses of tef anthers and microspores of various genotypes were investigated. Asymmetric division and multi cellular structures formation of the culture ms were observed after 9 and 10-15 days of culture, respectively. Formation of embryo like structure (ELS) and induction of 2 mm calli were obtained for various genotypes after 3 and 4 weeks of culture, respectively. However, in the anther derived calli none of these were able to regenerate in the various regeneration media. Factors affecting microspore culture in tef are discussed.

Anther culture is easy and need limited facilities compared to microspore culture. However, separating each anther from the hermaphrodites tef flower is both time consuming and pain staking. This is because the culture of ovaries with the anthers (easier than isolating the anthers) totally changed the response of the cultured explants to gynogenic response in this culture condition. In most cases, the ovaries enlarged and the anthers degenerated. Besides, in anther culture, embryogenesis may be induced on the anther walls or somatic tissues of the tapetum may not be clearly distinguished from the gametic embryogenesis. To avoid this problem, the culture of microspores becomes necessary although the procedure needs very fine tissue culture laboratory and other facilities such as micro blenders, centrifuges, fine sieves, as much as many various sizes disposable pippets and the like. The number of haploids from androgenesis is largely higher than using the anthers. However, many species including tef are found to be recalcitrant for this culture system. That is why gynogenesis is taken as an alternative approach, although the number of haploid production is much less compared to microspores. Albino plants from androgenesis studies is commonly reported in many species and it was observed during tef androgenesis, however, they are not found in gynogenesis of many plant species. The need for the refinement of the developed protocol of both anther and microspore culture is obvious since it requires further work especially in finding the appropriate regeneration media and appropriate hormones type and concentration to increase the number of ELS formation and growth of these embryos to embryonic callus stage.

8.4 Gynogenic culture

Isolated pistils, spikelets and panicle segments were compared for *in vitro* gynogenesis. Explants were cultured on MS media supplemented with 2 mg/l 2, 4-D and BAP for 2-4 weeks under light. Embryonic tissues were sprouted from the enlarged ovaries of the highly responsive explants of young panicles segments (14-17 cm) emerged out of the flag leaf sheath), after 10-15 days of inoculation. The effect of various factors for increasing the embryonic tissue induction was investigated. These include the optimum harvesting stage of panicle, suitable explant type, pre-treatment duration in cold, levels of 2, 4-dichlorophynoxyacetic acid (2, 4-D), and genotypic responses. All of the studied factors showed significant differences in the induction of embryonic tissues. The embryonic tissues from tef varieties and the ovary derived embryonic tissues from *E. mexicana* were successfully regenerated in the L3 regeneration medium and 182 and 6 plants

from tef and the wild species were obtained, respectively. L3 medium which differ from MS mainly with respect to ammonium nitrate, vitamin and amino acid content has previously been shown to be suitable for callus induction and regeneration of recalcitrant cultures for example microspore of cereals. This medium was also superior to microspore culture in pennisetum and sorghum immature embryo culture. However, this medium was unsuitable to tef genotypes studied here.

The gyno derived regenerated plants were transplanted in to the soil under green house condition and grown vigorously without any sign of albinos or other abnormalities. The indication of the flow cytometry analysis revealed that various levels of ploidies were generated in tef. Moreover, gametoclonal variation were identified for a number of quantitative and qualitative traits such as, grain yield, panicle weight, panicle length, flowering time, maturity, seed size and etc. DHs of rice derived from one genotype showed a range of phenotypes. Meiotic abnormalities that occurred in DHs may have been responsible for altered enzyme activities and agronomic performance of the DHs. However, the majority tetraploid regenerants obtained almost from the tested genotypes were not yet studied for any genetic changes. This needs further study by using molecular techniques such as AFLP as the homozygosity of gyno derived regenerants of onion was investigated or by field evaluation of successive generation.

In general, the many gametoclonal variation obtained from this study may be used for improvement of the crop.

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8.5 Transformation of tef

Foreign DNA delivery into the immature embryos of tef and establishment of stable transformation in tef by using both transformation methods (biolistic and *agrobacterium* mediated) were performed. The particle bombardment transformation of tef can circumvent the difficulties that occur when regeneration of plants from freshly excised immature embryos using *agrobacterium* mediated transformation. This can be resolved by using 2-7 days old embryonic calli for *agrobacterium* transformation instead of using the freshly isolated embryos.

The repeated bombardment experiments carried out at different times gave consistent results. This may indicates that, several of the factors that could have affected the efficiency of foreign gene delivery were regulated for tef biolistic transformation to achieve stable transformation. The

physiological state and morphological character of the cells (number of cells and age of development) were also optimized.

In any transformation system, only a small proportion of plant cells are transformed by the currently available delivery systems and, therefore, it is crucial to have a selectable marker to recognize the transformed cells and suppress the growth of wild type cells. Because sensitivity affects the recovery of transformed plants and varies widely among tissues and species, antibiotic sensitivity are usually determined in the initial stages of development of a plant transformation system.

Antibiotics such as hygromycin, paromomycin and herbicides like bialaphos are the current widely used selective agents. Our result indicated that hygromycin at lower concentration (5 µm) and bialaphos at higher concentration (50 mg/l) to be optimum for the growth of embryonic calli of tef. Besides in the present protocol both agro stop for at least three days but no resting periods were preferred to use as an appropriate method in order to reduce the risk of loosing the transformed cells at their younger stage and also not to holding the none transformed escapers. Five transgenic plants obtained from the present study were morphologically normal and were fertile. Thus, this successful and stable transformation technique developed for tef can be taken as a basis for future actual transfer of the gene of interest such as lodging resistant.

The major challenge identified in developing world especially in Africa was the persistent poor performance of agriculture, which is leading to a food and a poverty crisis. Where biotechnological approaches such as rapid multiplication of disease free plantlets such as banana in Kenya; use of new genetically improved pest-resistant cotton varieties by small farmers in south Africa; and use of new vaccines against animal disease in Kenya and Zimbabwe have contributed to the solution of specific problems, reduce the cost of pest control, and created new employment opportunities in towns and villages. Besides, the application of biotechnological advances could help countries to deal with the major challenges of feeding expanding populations from the existing land and water resources, and also alleviate poverty by stimulating the growth of bioscience based industries. The benefit from biotechnology such as double haploid breeding program, induction of male sterility, resistance to many biotic and abiotic challenges changed the current low productivity of many crops as green revolution did in the

past. New genotypes have been released using this technology. The benefit from this new technology is enormous which tef and other crops improvement programs may use.

A MAN'S STEPS ARE DIRECTED BY THE LORD.

HOW THEN CAN ANYONE UNDERSTAND HIS OWN WAY

. **Proverbs 20: 24**