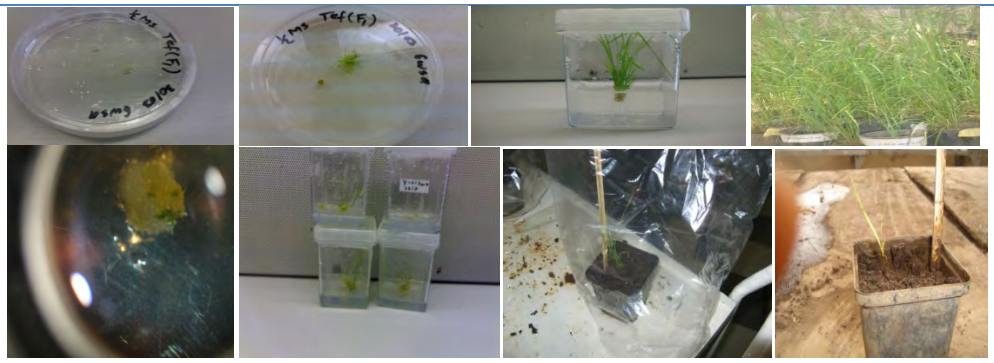
ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Regeneration of plants from unpollinated ovary cultures of Ethiopian wheat varieties (*Triticum* spp.) and embryo rescue cultures of F_1 hybrids of tef [*Eragrostis tef* (Zucc.) Trotter)] with its wild relatives



BY

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ALL THINGS WERE MADE THROUGH HIM, AND WITHOUT HIM WAS NOT ANYTHING MADE THAT WAS MADE

JOHN 1÷3

DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for degrees in any other University and all sources of material used for the thesis have been duly acknowledged.

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Table of Content Page

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ABBREVIATIONS

B_5	Gamborg *et al* medium
BAP	6-Benzylaminopurine
CEO	Cultured enlarged ovaries
CET	Cultured embryonic tissues
CF	Cultured florets
2,4-D	2,4-Dichlorophenoxy acetic acid
D	Days
Daac	Days after artificial crossing
DZARC	Debre Zeit Agricultural Research Center
ET	Embryonic tisssues
EO	Enlarged ovaries
EP	Embryo promoting
GA_3	Gibberellic acid
HARC	Holeta Agricultural Research Center
IAA	Indole -3-acetic acid
IET	Induced embryonic tissues
KIN	Kinetin
MS	Murashige and Skoog medium
N_6	Chu medium
NCE	Number of cultured explants
NSR	Number of shoots regenerated
NAA	Naphtalene acetic acid
PGR	Plant growth regulator
RET	Responsive embryonic tissues
SE	Somatic embryo
SD	Standard deviation
SR	Shoot regeneration
TCO	Total cultured ovaries
TET	Total embryonic tissues

List of Tables Page

List of Figures Page

List of appendices Page

ABSTRACT

Tef [*Eragrostis tef* (Zucc.) Trotter] and wheat (*Triticum* spp.) are the two top staple cereal crops in Ethiopia. Production constraints of cereal crops include fungal and viral diseases, insect pests, less quality and quantity of food grains, late maturation, lodging and others. For gynogenic cultures, unpollinated ovaries were excised from four varieties of wheat (*Triticum* spp.), two varieties from each of durum and bread wheat namely Yerer, Ude, Simba and Galema respectively. Light and dark culture conditions, durations of cold pretreatment at 4 ^{o}C, stages of harvesting, seasons, genotypes, types of media and compositions of the media affected induction of direct embryogenesis. ANOVA has shown that genotypes, stages of harvesting, types of media, combinations of PGRs (2,4-D and KIN) and durations of cold pretreatment at 4 ^{o}C significantly ($P \leq 0.05$) affected formation of embryonic tissues independently. Developmental stages of the donor plants were critical step in gynogenic response of cereal crops. Developmental stages of wheat spikes were identified and stage II was taken as the best stage for induction of embryonic tissues. Furthermore, light culture conditions, 30 g/l of maltose, MS medium, and 15 days of cold pretreatment were the best conditions for induction of embryonic tissues. From 12 combinations of 2,4-D and KIN supplemented in MS basal medium, 1 mg/l of each of 2,4-D and KIN was found to be the best combination for induction of embryonic tissues for varieties Yerer (35.0 %), Simba (26.6%) and Ude (13.3 %). Eleven different PGR combinations were used for shoot regeneration, 0.1 mg/l of NAA + 1 mg/l KIN was the PGR combination where the embryonic tissues of all varieties regenerated into shoots. The highest frequency of shoots were regenerated from the cultured embryonic tissues of varieties Simba (41.3 %) and Yerer (41.6 %) at 0.1 mg/l 2,4-D. From a total of 14,524 cultured ovaries, 1100 embryonic tissues (7.6 %) and 75 regenerants were obtained. The average percentage of embryonic tissues and regenerants were 9.0 % and 1.1 % from 3,444; 9.8 % and 0.55 % from 4,732; 5.6 % and 0.17 % from 2,988; 4.7 % and 0.12 % from 3,360 cultured ovaries for varieties Yerer, Simba, Ude and Galema respectively. In embryo rescue cultures, 8 combinations from 2 wild species of tef: *E. pilosa* (30-5), *E. pilosa* (37 80 82) and *E. curvula* and 4 domesticated varieties of tef: *E. tef* cv. Kaye Murri, *E. tef* cv. DZ-Cr-387, *E. tef* cv. DZ-Cr-37 and *E. tef* cv. DZ-01-196 were taken. Florets were cultured in MS medium supplemented with 6 different concentrations of 2,4-D which were excised from panicles after six days of artificial crossing, 1 mg/l 2,4-D and 0.1 mg/l of 2,4-D were found to be the best 2,4-D concentrations for induction of somatic embryos from the cross of *E. pilosa**Kaye Murri (46.7 %) and *E. pilosa**DZ-Cr-37 (13.3 %) respectively. From a total of 635 cultured florets of F_1 hybrids, 21 somatic embryos of F_1 hybrids were obtained . Of which 19 somatic embryos from the cross of *E. pilosa* (30-5)*Kaye Murri and 2 somatic embryos from the cross of *E. pilosa* (30-5)*DZ-Cr-37 were obtained. Fourteen somatic embryos (73.7 %) and 1 somatic embryo (50 %) were regenerated in half MS medium without plant growth regulators respectively. Twenty four plantlets of F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri were successfully transferred into pots. A total of 442 single plantlets were regenerated at maturity that were uniform, normal, fertile and no abberant plants were obtained. The plantlets showed inherited characteristics from both parents. It is recommended that the temperature of the growthroom and glasshouse should be adjusted for better survival of plantlets. Appropriate focus and facilities should be given for crossing of tef in order to avoid its bottlenecks with modern biotechnology.

Key words/phrases: Embryogenesis, embryo rescue, embryonic tissue, *Eragrostis tef*, ovary culture, plantlet, somatic embryo, *Triticum* spp.

1. INTRODUCTION

Cereal crops belong to the grass family Poaceae. Poaceae is the fourth largest family of the flowering plants. Poaceae is divided into the subfamilies of Panicoideae, Oryzoideae, Bambusoideae, Pooideae, Chloridoideae and Arundinoideae (Kellogg, 1998). According to this author, Poaceae includes all the major cereals such as wheat, maize, rice, sorghum, barely, oat and most of the minor grains, such as rye, common millet, finger millet, tef and many others that are less similar. It also includes economically important species, sugarcane. Understanding the grass family is thus central to understand the crop that feed the world. Therefore, continuous improvement of cereal crops is not questionable to feed the ever-growing population.

Some of the major production constraints of cereal crops that pose a serious threat to global food security are fungal and viral diseases, insect pests, less quality and quantity of food grains, late maturation, lodging, lack of adaptation to the ever changing phenotypic plasticity, unique pollination habit like tef, small size of the floral parts and others (Stallknecht *et al*., 1993; Seyfu Ketema, 1997; Likyelesh Gugsa, 2005; Jauhar, 2006; Yu *et al*., 2007). Moreover, cereal production faces growing challenges due to increasing human population and changing their nutritional requirements from time to time.

Cereals provide the major calories consumed by human. In Ethiopia, tef has as much, or even more, food value than the major grains. Tef grains are reported to contain 9-11 percent protein, an amount slightly higher than in normal sorghum, maize, or oats (Melak Haile Mengesha, 1965; Endeshaw Bekele and Lester, 1981; Geremew Bultosa, 2007). However, according to the report of Alemayehu Areda (1995), tef grain protein was in the average range of 11.2-12.5 percent. Wild relatives of modern crop species have survived for millions of years using natural genetic defence to endure biotic and abiotic factors. These wild relatives represent a valuable source of under-utilized genetic variation that is available to plant breeders and represent an invaluable source of genetic information for modern genomic research initatives (Raupp and Manhattan, 2007).

Ethiopia is the center of origin for crops like anchote [*Coccinia abyssinica* (Lam) Cogn.], chat (*Cata edulis* Forsk), coffee (*Coffea arabica* L.), and endod (*Phytolaca dodecandra* L'Herti), enset and tef. From the cereals, tetraploid wheat is a prominent crop for which the country is recognized as the secondary center of diversity (Abebe Demissie and Giorgis Habetemariam, 1991). Ethiopia is one of the

seven primary geographic centers of domestication, Vavilov`s centers of domestication, in the world (Smale *et al*., 1996).

Tef and wheat belong to class: Liliopsida, order: Poale and family: Poaceae. However, they diverged into different subfamilies. The former belongs to subfamily: Chloridodeae, genus: *Eragrostis* and the latter belongs to subfamily: Pooideae, genus:*Triticum* (Smale *et al*., 1996). They are predominantly selfing and annual crop plants. Depending on cultivar and year, in wheat, outcrossing rates of up to 10 % were observed on some varieties (Waines and Hegde, 2003). Outcrossing rates of 0 to 2 % are commonly accepted. However, in tef [*Eragrostis tef* (Zucc.) Trotter], the degree of outcrossing is very low (up to 1 %) (Stallknecht *et al*., 1993). Tef [*E. tef* (Zucc.) Trotter] is originated, dominantly cultivated, a C_4 and major staple food crop in Ethiopia. Except for few studies, tef has not been enjoyed from the benefited from modern tools of biotechnology (Teklu Yifru and Hailu Tefera, 2005; Yu *et al*., 2007). However, wheat is a C_3 crop plant, originated in the Fertile Crescent, and one of the major staple food crops cultivated worldwide (Waines and Hegde, 2003).

Eragrostis comprises more than 350 species, mainly found in tropical and subtropical regions, of which 14 are said to be endemic to Ethiopia. Tef is the only *Eragrostis* species cultivated for its grain. Humans sometimes eat the grains of several forage species mainly as famine food, particularly *Eragrostis cilianensis* (All.) F.T.Hubb., *Eragrostis ciliaris* (L.) R.Br., *Eragrostis curvula* (Schrad.) Nees, *Eragrostis cylindriflora* Hochst., *Eragrostis gangetica* (Roxb.) Steud., *Eragrostis pilosa* (L.) P. Beauv., *Eragrostis tremula* Steud. and *Eragrostis turgida* (Schumach.) De Wild (Teklu Yifru and Hailu Tefera, 2005).

In Ethiopia, tef and wheat are adapted to wide range of environment and are presently cultivated under diverse agro climatic conditions. They can be grown from sea level up to 2800 meters above see level, under various rain fall, temperature, and soil regimes (Seyfu Ketema, 1993; Evert *et al*., 2008).

Tef is a good source of minerals, particularly Ca and Fe (Melak Haile Mengesha, 1965). Cattle prefer tef straw over straw of other cereals, and its quality is comparable to good natural pasture (Seyfu Ketema, 1997).

Allopolyploidization process has marked the evolution of cereals such as wheat, oat, and tef. These important food crops are evolved through the combination of two or more ancestral grass species brought to thrive together harmoniously within the same cell through thousands of years of natural and human-

driven selection (Mergoum, 2004). Wheat genetics is more complicated than that of most other domesticated species. It contains more than 1600 Mbp of DNA. Some wheat species are diploid, but many are stable polyploids. Their basic chromosome number is x =7. The genus *Triticum* is divided into three ploidy levels (diploid 2*n* = 2*x* = 14, tetraploid 2*n* = 4*x* = 28 and hexaploid 2*n* = 6*x* = 42) (Fernandes *et al*., 2000; Waines and Ehdaie, 2007). However, tef [*E. tef* (Zucc.) Trotter] is naturally an alloteraploid (2n = 4x = 40) and an octaploid level (2n = 8x = 80) was obtained through gynogenic culture, comparatively has small genome size 730 Mbp and basic chromosome number (x = 10) (Likyelesh Gugsa *et al*., 2001; Ingram and Doyle, 2003; Likyelesh Gugsa *et al*., 2006; Yu *et al*., 2007).

Eventhough conventional breeding has been alleviating some of the production constraints, its mechanisms of improvement do not go in parallel with the ever-increasing population growth. Thus, biotechnology, genetic engineering in particular, accelerates the improvement of production constraints of cereal crops and finding solutions for the constraints not yet alleviated by conventional breeding (Jauhar, 2006).

Genetic engineering is used to engineer new traits into plants. The successful deployment approaches to combat insect pests and diseases of important crops like rice (*Oryza sativa*), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), barely (*Hordeum vulgare* L.) and cotton is a remarkable accomplishment (Jauhar, 2006).

In vitro regeneration of plants is one of the pre-requisites for successful genetic transformation and hence an efficient *in vitro* regeneration protocol is necessary for different explants. Biotechnology helps researchers to breach the crossing barriers that once limit conventional breeding (Smale *et al*., 1996).

Haploids can be induced either *in vivo* or by various *in vitro* methods using female and male parts of the plant. *In vivo*, haploids can be produced via semigamy, polyembryony, chromosome elimination, gynogenesis and androgenesis at extremely low frequencies. *In vitro*, haploids can be induced via cultured anthers or microspores (androgenesis), ovules or ovaries (gynogenesis) or via distant hybridization using pollen from another species (Forster *et al*., 2007).

Production of haploid plants *in vitro* is the fastest and the only easy method for producing homozygous lines. *In vitro* culture of unpollinated ovaries and ovules have been successfully applied to many plant species that are not amenable to androgenesis. Many of the problems associated with androgenesis such as albinism, inviability and recalcitrant could be overcome by culturing unfertilized ovule/ovary/ flower

buds. They are used to induce a haploid cell of the female gamete to develop into a haploid sporophyte (Shivanna and Mohan Ram, 2005; Satyaranayana, 2007; Jauhar *et al*., 2009).

Wheat cultivars developed from doubled haploids have been released for cultivation in Canada, China, Europe, and Brazil (Jauhar *et al*., 2009). In Ethiopia, double haplod plants were obtained from tef and they are under investigation in HARC (Likyelesh Gugsa pers. Communication). Haploid production using *in vitro* ovule cultures has long been recognized as an important tool to produce haploid and homozygous double-haploid plants for genetic studies and plant breeding programs (TÖpfer and Steinbiss, 1985).

The occurrence of postfertilization disorders constitutes a major hurdle to stable hybrid embryo development in wide crosses. Implementation of molecular and cellular genetic methods in a breeding program requires an efficient regeneration system from somatic embryogenesis. The application of tissue culture techniques, particularly in the area of embryo rescue, has had a major impact on the maintenance and development of hybrid embryo from wide crosses. Embryo rescue techniques are directed towards obtaining more efficient survival of embryos in situations where very immature embryos are to be cultured (Niiami *et al*., 1995; Raghavan, 2003).

2. LITERATURE REVIEW

Human efforts in plant breeding have been continuum for the past 10,000 years. Breeding programs have been trying to produce elite cultivars that can adapt to a range of environments without compromising agronomic performance, grain quality or disease resistance. The combination of existing knowledge and resources with modern biotechnology and functional genomics is providing the opportunity to study the genetic, biochemical and physiological basis of these complex traits. Current efforts aim to address the major challenge of capturing the information from both wheat and model organisms, such as rice and *Arabidopsis* (Jauhar, 2006; Dubcovsky and Dvorak, 2007).

2.1 WHEAT (*TRITICUM* SPP.)

2.1.1 Morphology and chemical compositions

Wheat plants develop through distinct vegetative and reproductive growth stages. The vegetative stage begins within two weeks of sowing when the coleoptiles emerge from the soil. The first leaf soon emerges through a vent at the tip of the coleoptiles, with other leaves emerging in succession. The shoot is a short rhizome bearing several leafy culms and tillers which may each grow to over a meter in height. After the first few leaves have appeared, tillers grow from buds at the nodes of the stem, at or below the soil surface. Following tillering, the wheat plant enters a period of rapid growth known as stem extension. In this phase, both the stem and leaf-sheath elongate rapidly. The upper most leaf is called the flag leaf (Peterson, 1965).

The vegetative growth stages are followed by a period of reproductive growth. The top internodes within the sheath of the flag leaf bear the spike and are the last internodes to lengthen. As the spike grows in size, it inflates the sheath of the flag leaf, which is referred to as the boot. The emergence of the spike from the boot is called heading (Peterson, 1965). The basal spikelets are often entirely sterile. Each spikelet contains three florets. Each floret has three stemns and a pistil. The pistil consists of an ovary, two short styles and a branched feathery stigma. Stigmas are receptive for 2 to 13 days after anthesis and are most receptive for the first 2 to 5 days (Waines and Ehdaie, 2007).

Wheat varieties can be differentaited by glume color, awn color, spike density, seed color, seed shape, glume pubescence, awn size, straw strength, stem structure, spike fertility, head width, and spike length are some of the characters with high genetic variation (Efrem Bechere *et al.*, 1996).

The wheat grain contains starch (60-70 %), protein (10-17 %), fiber (2-2.5 %), fat (1.5-2.0 %), sugar (2-3 %), water (12 %), and mineral matter (1.5-2.0 %) (Yadav *et al*., 2009).

2.1.2 Origins and distribution

Fertile Crescent (South east Turkey) is one of the centers of origin of agriculture and the region at which the diploid grass progenitors of durum and bread wheats orginated. Wheat spreads with extension of human settlement and cultivation practices but probably remained confined within Afro-Eurasian landmass (Salamini *et al*., 2002; Waines and Hegde, 2003).

Tetraploid wheat (*Triticum turgidum* L. var. Durum) is an indigenous species to Ethiopia. Ethiopian farmers have cultivated durum wheat since time immemorial. Due to an amazing wealth of genetic variability for durum wheat, Ethiopia is also recognized as the center of genetic diversity for this crop (Smale *et al*., 1996). The genus *Triticum* in Ethiopia is represented by seven species including *T. diccocum*, *T. durum*, *T. turgidum*, *T. polonicum*, *T. aestivum*, *T. compactum* and *T. pyramidale* (Abebe Demissie and Giorgis Habetemariam, 1991; Kassahun Tesfaye, 2000).

2.1.3 Domestication

Cultivation, repeated harvesting and sowing of the grains of wild grasses led to the domestication of wheat through selection of mutant forms with tough ears, larger grains, and a tendency for the spikelets to stay on the stalk until harvested. Because of the loss of seed dispersal mechanisms, domesticated wheat has limited capacity to propagate in the wild (Waines and Hegde, 2003).

All of the wild and domesticated wheat may be classified into three groups. The diploid *Triticum* spp. (AA, 2n = 2x = 14), the tetraploid *Triticum turgidum* (AABB, 2n = 4x = 28), includes emmer and durum, the hexaploid *Triticum aestivum* (AABBDD, 2n = 6x = 42) (Table 1). The source of A genome is generally believed to be *Triticum urartu*, the source of B genome is disputed, and the source of D genome is *Triticum tauschii* (Smale *et al*., 1996; Salamini *et al*., 2002). However, Waines and Ehdaie (2007) demonstrated that the wheat genome B came from diploid *Aegilops* species. The evolution of modern polyploid wheat varieties was the domestication of wild emmer (*T. dicoccoide*). Wild emmer is the result of hybridization between two diploid wild grasses, *T. urartu* and a wild goat grass such as *A. searsii* or *A. speltoides*.

Common wheat, bread wheat, orginated through interspecific hybridization of three diploid species of grass, each of which donated a genome (Zohary, 1969). The diploid A, B and D progenitor species of bread wheat diverged from a common ancestor approximately 3 MYO (Bhalla, 2006).

Table 1. Common name, Biological species name, Ploidy level and Genome of some wheat species.

Common name	Biological species name	Ploidy level	Genome
Wild einkorn	*Triticum monococcum* ssp. *boeoticum* Boiss.	Diploid	AA
Cultivated einkorn	*Triticum monococcum* ssp. *monococcum*	Diploid	AA
Wild Triticum urartu	*Triticum urartu* Tuman	Diploid	AA
Wild A. tauschii	*Aeiglops tauschii* Coss	Diploid	DD
Wild emmer	*Triticum dicoccoides* Aschers	Tetraploid	AABB
Cultivated emmer	*Triticum dicoccum* Schubl	Tetraploid	AABB
Durum (hard) wheat	*Triticum durum* Desf.	Tetraploid	AABB
Wild Timopheev`s wheat	*Triticum timopheevii* Zhuk ssp. *araticum* Jakubz.	Tetraploid	AAGG
Cultivated Timopheev`s wheat	*Triticum timopheevii* Zhuk ssp. *timopheevii*	Tetraploid	AAGG
Spelt wheat	*Triticum spelta*	Hexaploid	AABBDD
Bread wheat or Common wheat	*Triticum aestivum* L.	Hexaploid	AABBDD

Source: Ethiopian and Eritrea flora book (1995) and Salamini *et al*. (2002).

2.1.4 Breeding objectives and methods

Wheat-breeding efforts have been focusing on producing new varieties with high yielding and improved disease resistance, nematode, fungal, pest, and/or viral infection. In addition, they should have tolerance to abiotic stresses such as, heavy-metal toxicity, drought, cold tolerance and numerous grain quality attributes that affect baking and other uses of the final product (Tesfaye Tesemma and Jamal Mohammed, 1982; Jauhar, 2006; Hulshof *et al*., 2007). Moreover, fix spike characteristics and increased grains per spike; produce lodging resistance and high gluten containing varieties; increase nutrient use efficiency, especially nitrogen and phosphorus; and introduce semi-dwarf cultivars to increase yield are some breeding objectives of wheat (Tesfaye Tesemma and Jamal Mohammed, 1982; Getachew Belay and Tesfaye Tesemma, 1990; Raupp and Manhattan, 2007). However, the existing conventional breeding

procedures are time consuming and may take 10 or more years to transfer a trait from a donor species into a crop cultivar (Jauhar, 2006).

Selection based on the morphology of wheat, crossing of wheat with other domesticated varieties. Moreover, recurrent selection, mass selection, and good management have been also the breeding methods to address the breeding objectives (Getachew Belay and Tesfaye Tesemma, 1990; Jonsson, 1995; Salamini *et al*., 2002; Raupp and Manhattan, 2007).

2.1.5 Cytogenetics

Triticum is classified into three ploidy groups, with basic chromosomes (x = 7). The diploid wheats have 14 chromosomes (2n = 2x = 14), the tetraploids have 28 chromosomes (2n = 4x = 28) and the hexaploid species contains three different ancestral genomes, designated A, B, and D (2n = 6x = 42) (Fernandes *et al*., 2000; Waines and Ehdaie, 2007).

According to the report of Getachew Belay *et al*. (1994), no aneuploidy was encountered in Ethiopian genotypes of *Triticum turgidum*. The authors demonstrated that tetraploids of Ethiopian genotypes had pairs of satellited, eight median and four submedian chromosomes. *Triticum aestivum* has submedian chromosomes (Raupp and Manhattan, 2007). It possesses 21 pairs of homologous chromosomes. These homologous chromosomes are classified into seven homoeologous groups. Each group contains one pair of chromosomes from the B, A and D genomes (Sears, 1954). Jauhar *et al*. (2009) demonstrated that the A and D genomes of wheat are more closely related to each other than either one is to the B genome.

Meiosis stage is extremely susceptible to low temperatures, under 4 °C or high temperatures, over 25 °C (Smale *et al*., 1996). Meiotic chromosome pairing in *Triticum aestivum* is controlled by genetic systems that can promote or reduce pairing. The pairing of homoeologous chromosomes is prevented principally by the activity of a single locus (*ph*) located on the long arm of chromosome 5B (Riley, 1972). The availability of a variety of aneuploids and the *ph* mutants *(ph1* and *ph2)* in bread wheat allowed chromosome manipulations leading to the development of alien addition/substitution lines and the introgression of alien chromosome segments into the wheat genome (Gupta *et al*., 2005).

The total wheat genome is in excess of 16,000 mega bases in size, and approximately 80 % of it consists of repetitive, non-coding, DNA sequences. It can tolerate major chromosomal rearrangements and deletions. Most of the non-repeated sequences of the wheat genomes are about 1000 nucleotides long and

interspersed between the repeated sequences. It is undoubtedly one of the largest and most complex genomes of all crop species (Francki and Appels, 2002).

2.1.6 Ecology and agronomy

In Ethiopia, wheat is traditionally grown by small scale farmers on heavy black clay soils at altitude ranging from 1800-2800 meters above sea level. It is produced in a wide range of climate and geographic regions (Tesfaye Tesemma and Getachew Belay, 1991). Durum wheat is grown exclusively under rain fed conditions (Tesfaye Tesemma and Getachew Belay, 1991). However, bread wheat is still cultivated under rain fed and irrigation in Mali, Nigeria, Chad and the Central African Republic (Smale *et al*., 1996; Yesayan *et al*., 2009).

Monocultures of modern cereal crops are popular due to technical and organizational reasons. They are easier to manage interms of crop husbandry and marketing. However, in this monoculture, chemical protection of the crops is compulsory to reduce yield loss due to diseases, pests, and sometimes weed infestation. Tillage and crop rotation are agricultural practices which are known to cause changes in soil microbial communities and soil management (Padulosi *et al*., 1995; Raupp and Manhattan, 2007).

2.1.7 Production status and its constraints

The regions of Ethiopia that are main producers of wheat are Shewa, Gondar, Gojam, Wello, Arsi and Bale. Wheat has been and continues to be one of the most important cereal crops in the country in terms of both area under cultivation and production. In 2007/2008 Ethiopian meher season, the area under wheat production was estimated at 1, 479, 287.16 ha with an average production of 1.833 tonnes per hectare. It was the largest area covered next to tef and maize. In terms of average production, wheat ranks second following maize (CSA, 2008). From 1948 to the present, bread wheat (*Triticum aestivum* L.) yields in the United Kingdom have increased by an average of 110 kg/ha each year (Austin, 1999). According to the reports of Ortiz (2008), the global annual harvested areas of bread wheat and durum wheat were on average 217 million ha, producing 621 million t (Table 2).

Table 2. Area productivity of wheat in selected regions of the world 2004-2006.

Region	Area Million ha	Yield t/ha	Production Million t
European Union	26	5.3	137
East Asia	23	4.3	98
South Asia	38	2.5	97
North America	31	2.8	88
South America	9	2.4	22
Middle East and NorthAfrica	27	2.3	61
Rusia and East Europe	31	2.2	69
Central Asia and Caucasus	15	1.4	22
Australia and New Zealand	13	1.5	19
Sub Saharan Africa	4	2.3	9
World	217	2.9	621

Source: Ortiz (2008).

Estimates of the amount of wheat production lost owing to plant diseases caused by viruses and fungi vary between 10-25 %. Fusarium head blight, stem rust, leaf rust and stripe rust are diseases of bread wheat (Manyingerew Shenkut *et al*., 2006). In comparison, leaf and stripe rusts have caused minor crop losses than stem rust. Historically, stem rust caused a major world wide problem except East Africa. Moreover, wheat is used as a food plant by the larvae of some Lepidoptera species and aphids (*Diuraphis noxia*) (Smith *et al*., 2004). Abundances of rodents around wheat fields and toxicity of aluminium are major threats of wheat production (Mujeeb *et al*., 1995; Raupp and Manhattan, 2007).

2.1.8 Economic importance

In Ethiopia, the grain of wheat is consumed in different forms such as leavened bread, pancakes, macaroni and spaghetti, biscuits and pastries. The most common Ethiopian recipes are Dfo-Dabo (Ethiopian loaf of bread) which is usually prepared during holidays and joyful events, hambasha (bread mainly from Northern Ethiopia), kitta (unleavened bread), dabo-kolo, injera (thin bread), nifro, kolo, kinche and chechebsa (Tesfaye Tesemma and Getachew Belay, 1991; Efrem Bechere *et al*., 2000).

2.1.9 Biotechnology and its application

Biotechnology aimed at improving crops by focusing on three main areas: *in vitro* culture, genetic transformation and molecular markers analysis (Aulinger, 2002). Exploring the application of biochemical and molecular markers to enhance the detection of alien introgressions is one goal of recent biotechnology research to obtain doubled haploid plants. They offer complete homozygosity and phenotypic uniformity in one generation (Jauhar, 2009).

2.1.9.1 Molecular marker

Restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and random amplified polymorphic DNAs (RAPDs) are the predominant marker systems that have been used to develop a variety of wheat and tef genetic maps (Mulu Ayele and Nguyen, 2000; Francki and Appels, 2002; Kebebew Assefa *et al*., 2004; Ameha Yaekob, 2005; Yu *et al*., 2007; Burger *et al*., 2008). They have been used extensively in genetic characterization and identification of loci controlling traits, indirect selection of traits of importance, valuable tools for crop improvement and help researhers to understand complex traits. Moreover, they measure genetic diversity, determine phylogenetic relatedness, applied for linkage analysis, used for marker assisted selection, used for identification of alien chromosomes, estimation of genome homology and cultivar identification.

2.1.9.2 Tissue culture

Plant tissue culture broadly refers to the *in vitro* cultivation of plants, seeds and parts of the plants such as leaf, immature and mature embryos, flower buds, anthers, microspores, ovaries, tissues, single cells, protoplasts and others. Tissue culture technology can potentially regenerate species of any plant in the laboratory. Improved *in vitro* tissue culture systems are needed to facilitate the application of transgenic technology to the improvement of cereal germplasms (Aulinger, 2002; Zhang *et al*., 2004). *In vitro* androgenesis and gynogenesis are very significant technology for production of double haploids in any genotypes (Liu *et al*., 2002; Raupp and Manhattan, 2007).

Tissue culture can also rescue hybrid embryos showing failure of endosperm development which lead to the death of potentially viable hybrid embryos. This can be overcome by culturing the developing embryo on artificial medium (Baum *et al*., 1992; Shivanna and Mohan Ram, 2005; Sheibani *et al*., 2007). It is also used to produce haploids. For instance, wheat crossed with *Hordum bulbosum*, sorghum, maize and pearl millet. The centeral aim of wide crossing programs in plant breeding is the introduction of novel characteristic into elite cultivars (Baum *et al*., 1992).

2.1.9.3 Gynogenic haploidization

Gynogenesis is the process of regenerating haploid plants from ovaries, ovules, flower buds, spikelets, spikes and panicle cultures (Campion *et al*., 1992; Castillo and Cistue, 1993; Kumlehn and Nitzsche, 1995; Thomas, 2004; Likyelesh Gugsa, 2005; Sheibani *et al*., 2007; Bhat and Murthy, 2008). San (1976) first developed gynogenic haploids from the ovary cultures of *Hordeum vulgare*. Successful results of *in vitro* gynogenesis to produce haploids and double haploids have been reported in barely (*Hordeum vulgare L*.) (San, 1976; Castillo and Cistue, 1993), wheat (*Triticum turgidum* L.) (Alaoui *et al*., 1998; Slama-Ayed and Slim-Amara, 2007), *Beta vulgris* L. (Bossoutrot and Hosemans, 1985), Tef (*Eragrostis tef*) and *Eragrostis mexicana* (Likyelesh Gugsa *et al*., 2006), niger (*Guizotia abyssinica* (L.f) (Bhat and Murthy, 2008), and *Lolium multiflorum* (Kumlehn and Nitzsche, 1995). Gynogenesis may be the only efficient means of producing haploids in *Beta vulgaris*, *Allium cepa*, and *Gerbera jame* Sonii (Bhat and Murthy, 2008).

Gametic embryogenesis can be developed through wide hybridization, androgenesis, and gynogenesis. The regeneration of the gametic embryos into plants was improved by means of an indirect regeneration method, based on the induction of somatic embryogenesis prior to regeneration (Aulinger, 2002).

A three-step procedure was adopted for the induction of gynogenesis in two cultivars of mulberry (*Morus alba* L.). This includes *in vitro* flowering, inflorescence segment culture and isolated ovary culture. In the third step, that is, isolated ovary culture, the cultured ovaries burst and an embryo emerged from the ovary. The study confirmed that the gynogenic embryo emerged from the egg cell. Before the onset of division of the egg, all other cells of the embryo sac degenerated in the majority of ovules, but in exceptional cases the polar nuclei will be retained along with the dividing egg cell. The presence of the gynogenic embryo along with free-nuclear autonomous endosperm is the most important feature of the present investigation. Autonomous endosperm is formed from either the polar nuclei or secondary nucleus. In both cultivars used for the experiment, the percentage of ovaries showing proembryo induction during inflorescence segment culture is much higher than that of ovaries producing gynogenic plants during isolated ovary culture. This suggests the degeneration of some gynogenic embryos during the initial stages of induction (Thomas, 2004).

In vitro culture of unpollinated ovaries or ovules is usually employed when the anther cultures give unsatisfactory results for the production of haploid plants. Thus, it is used to overcome the problem of albino plant formation in anther culture; most efficient and reliable technique for the production of haploid and doubled haploid plants of some plant species; to produce male sterile line. However,

production of gynogenic haploids through female gametophyte still needs more refinement and also there are problems in dissection of unfertilized ovules and ovaries; has an inherent disadvantage of one ovary per flower to large number of microspores in one another (Satyanarayana, 2007).

Potential use of wheat in tissue culture studies have been reported using various explants such as immature embryos (Sears and Deckard, 1982), anthers (Dogramaci-Altuntepe *et al*., 2001), microspores (Liu *et al*., 2002) and unpollinated ovaries (Slama-Ayed and Slim-Amara, 2007).

2.1.9.4 Factors affecting ovary cultures

There are many factors affecting ovary cultures. The factors include genotypes and growth conditions of the donor plants, developmental stages of ovaries, compositions of nutrient media, culture conditions, differnt concentrations of sugars, seasonal effects, and pretreatment of donor materials (Sears and Deckard, 1982; Campion *et al*., 1992; Thomas, 2004; Mukhambetzhanov, 1997; Likyelesh Gugsa, 2005; Bhat and Murthy, 2008; Obert *et al.*, 2009).

Genotypes and growth condition of donor plants

Ovary development, anther response, callus induction and plantlet regeneration was influenced significantly by genotype and growth conditions (Campion *et al*., 1992; Doramaci-Altuntepe *et al*., 2001; Sibi *et al*., 2004; Thomas, 2004; Lin Shu *et al*., 2006; Slama-Ayed and Slim-Amara, 2007). From a total of 12,000 cultured unpollinated ovaries, 84 planlets were obtained (Slama-Ayed and Slim-Amara, 2007). Production of gynogenic haploid plants from ovary culture of *Allium cepa* L. was genotype dependent (Campion *et al*., 1992). The gynogenic response differed markedly between the two studied cultivars of mulberry: cv. K-2 was most gynogenic, with a maximum response 16%, whereas cv. S-1 exhibited very poor response (Thomas, 2004). In immature embryo cutures of wheat, variability was observed among the wheat genotypes tested for callus induction, regenerable callus formation, response to subculture, and plant regeneration potential (Sears and Deckard, 1982). In spike and immature embryo cultures of bread wheat 15 out of 17 genotypes were regenerated into plantlets (Lin Shu *et al*., 2006). In anther cultures of durum wheat, only 3 of the 10 genotypes produced green plants and from a total of 86,400 anthers cultured, 324 plants were obtained: 248 green and 76 albino. Genotype and growth condition significantly affected anther response, callus induction, and plant regeneration. Their interactions were also significantly affect anther response, callus induction and plant regeneration (Doramaci-Altuntepe *et al*., 2001).

Culture media and media compositions

Culture media that have been used for ovary culture so far are NN (Nitsch and Nitsch, 1969), N_6 (Chu, 1978), MS (Murashige and Skoog, 1962) and B_5 (Gamborg *et al*., 1968). They are made up of micro and macro nutrients, vitamins, sugars and growth regulators. The sugar can be sucrose or maltose. Auxins (2,4-D, NAA, MCPA, IAA), cytokinin (BAP, KIN) and gibberellic acid (GA_3) have been used in ovary cultures (Campion *et al*., 1992; Kumlehn and Nitzsche, 1995; Bhat and Murthy, 2008).

The media were solidified with 8 g/l agar and the pH was adjusted to 5.8 before autoclaving at 1.5-2 atm and 121°C for 15 min. All cultures were kg cm maintained at 25 ± 2°C under a 16 h photoperiod supplied by two Philips TL 40 W fluorescent tubes. Surface sterilization for rice panicle 20% Chlorox solution (commercial bleach containing 5.25% (w/v) NaOCl) for 20 min in disposable 60 x 15 mm plastic petridishes and kept in the dark. For callus ini tiation cultures have to be kept in dark (Tsuchya, 1992; Sibi *et al*., 2004).

A study of various components of the induction media based on that of Murashige and Skoog medium showed that 2,4-dichlorophenoxyacetic acid was necessary for callus formation and green plant regeneration (Alaoui *et al*., 1998). Shoots were initiated by reducing the 2,4-D from 1 mg/l to 0.1 mg/l and complete plants were regenerated by transferring to 2,4-D-free medium (Sears and Deckard, 1982). Kinetin and 2, 4-D were evaluated for callus induction of immature embryo culture of wheat (Jones *et al*., 2005) and for induction of embryonic tissues in ovule cultures of *Lolium multiflorum* (Kumlehn and Nitzsche, 1995) and in squash ovule cultures (Shalaby, 2007), 1 mg/l from each of kinetin and 2, 4-D was the optimum for the induction of embryonic tissues, the immature embryos formed the highest amount of callus on MS media supplemented with 2, 4-D and Kinetin while mature embryos formed the highest amount of callus with 2, 4- D (Yadav *et al*., 2009). Bhat and Murthy (2007) reported that 30 g/l sucrose was used as optimum sugar source for induction of embryonic tissues from ovule cultures of niger. In spike and immature embryo cultures of bread wheat, the optimum medium for callus induction was Murashige and Skoog (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and the optimum green plantlet differentiation medium was aslo MS medium (Lin Shu *et al*., 2006). According to these authors, 80 g/l sucrose was used the optimum carbon source for plant regeneration. kinetin showed no effects, supplements of 2,4-D (1~10mg/1) or IAA (50mg/1) to the basal media induced calli from seedling roots of einkorn, emmer and common wheats, and from stem pieces of common wheat. Callus growth was also vigorous when 2,4-D (0.5~2.0mg/1) was added. Root formation from callus took place in all kinds of tested media, except those containing no growth factors or

supplemented with 2,4-D at high concentrations (1~5mg/1). No growth factors were found to be specifically effective on shoot differentiation (Shimada *et al*., 1969).

The yeild of gynogenic embryo increases when 100 g /l of sucrose was used. Ovary culture gave best response in the presence of NAA than 2,4-D as auxin source (Campion *et al*., 1992). In mature embryo culture of bread wheat, MS medium supplemented with 10 mg/l 2-4-D and 30 g/l sucrose gave the optimum calli. The calli of the control treatment of all cultivars were transferred to regeneration medium supplemented with 0.5 mg/l indole acetic acid (IAA) and 1mg/l BAP and under different levels of ABA (0, 2, 4, 6 and 8 mg/l). After one month, it was observed that cultivars showed different rates and different abilities of regeneration (Fazelienasab *et al*., 2004).

Pretreatment of donor plants and develpmental stages of ovaries

Up to 9 days of cold pretreatment were tested to improve gynogenic culture of tef pistilate, however, there was no success. Embryonic tissues emerged out of the enlarged ovaries at their micropylar end and often entirely overgrew the pistil within 2 weeks. Panicle and spikelet cultures of tef were induced embryonic tissues without passing the callus phase, plant regeneration through direct embryogenesis (Likyelesh Gugsa *et al*., 2006). Cold pretreatment of panicle and spikelet culture at 4 ^{o}C can improve the formation of gynogenic tissues, 36 h was the best. In durum wheat, pretreatment at 4^{o}C for 14 days gave the highest response of ovary development and callus induction. The highest regeneration frequency was obtained when the microspore population was in the late mononucleate to binucleate stage (Slama-Ayed and Slim-Amara, 2007). Cold steress pretreatment of tassels with microspores at a uni- to early bi-nucleated developmental stage were removed from the plants wrapped in their leaf whorl and wrapped in moist paper towels and aluminium foil. The material was kept at 8 to 9 °C for 10 to 14 d for a cold stress treatment (Aulinger, 2002). A thermal pretreatment of 5-15 d at 5 °C and an initial period of darkness (2-5 weeks) followed by light (16 h daily) were also favorable for gynogenesis. All plants formed were green and chromosome counts in root tips of regenerated plants showed the haploid number n = 14 (Alaoui *et al*., 1998).

The stage at which the explants cultured is critical step for gynogenic response (Thomas, 2004; Likyelesh Gugsa, 2005). The mode of development of gynogenesis in Mulbery is via direct embryogenesis (Thomas, 2004). *E. Mexicana* florets at bi or tricellular pollen stage (2-1 days before anthesis) or from tef varieties, DZ-01-196 at the bi-cellular pollen stage continued to enlarge and eventually formed friable callus. Morphogenic expression leading to green plant formation in unfertilized ovary culture (gynogenesis) was studied in durum wheat (Alaoui *et al*., 1998). For gynogenic response of

durum wheat, the spikes were harvested when microspores were at bi- or trinucleate stage (Sibi *et al*., 2004).

Types of explants

In *E. mexicana*, regeneration of six green plants resulted from culture of 121 non-pollinated immature pistils. In the allotetraploid crop species tef, however, only callus and root formation was obtained by this method. By contrast, immature spikelets and panicle segments of *E. tef* proved amenable to gynogenic plant regeneration. Upon step-wise optimization of the protocol, efficient plant formation was achieved in all three cultivars tested. In cv. DZ-01-196, culture of 1305 immature spikelets resulted in formation of 159 green plants. Ovary culture of onion gave better number of embryos than flower culture (Campion *et al*., 1992).

The percentage of ovaries showing proembryo induction during inflorescence segment culture is much higher than that of ovaries producing gynogenic plants during isolated ovary culture. This suggests the degeneration of some gynogenic embryos during the initial stages of induction (Thomas, 2004).

2.1.9.5 Applications of haploidization

Production of haploid plants are useful for basic researches such as genetic mapping and analysis, induction of mutation, transformation, protoplast culture and somatic hybridization, biochemical and physiological studies, artificial seed production and germplasm storage. Moreover, it is essential for cytogenetics research such as production of aneuploids; determination of the nature of ploidy; evaluation of the origin of chromosomes and elucidate the genetic control of chromosome pairing inherently present in allopolyploids such as bread wheat, durum wheat, and oats (Mukhambetzhanov, 1997; Liu *et al*., 2002; Bhat and Murthy, 2008; Jauhar *et al*., 2009).

2.2 TEF [*ERAGROSTIS TEF* (ZUCC.) TROTTER]

2.2.1 Morphology and chemical compositions

Tef is a fine stemmed tufted annual grass. The plant has the appearance of a bunch grass, having large crowns and many tillers. The inflorescence is an open panicle and produces small seeds; 1,000 seeds weigh 0.3 to 0.4 g. The roots are shallow and develop a massive fibrous rooting system. Plant height of tef varies within the ranges of 50-200 cm, dependent upon cultivar type and growing environments (Stallknecht *et al*., 1993**)**. Stem (culm) is usually erect, simple or sparsely branched. Florets are bisexual. Each floret has 3 stamens. Anthers are up to 0.5 mm long and 2-celled. Ovary is superior with two stigmas. The fruit is a caryopsis (grain), ovoid to ellipsoid, yellowish-white to deep brown. Tef grain comes in a range of colors from milky white to almost dark red, but its most popular colors are white, red, and brown (Stallknecht *et al*, 1993; Tadesse Ebba, 1975).

The composition of whole tef grain per 100 g edible portion is water 11 g, protein 9.6 g, fat 2.0 g, carbohydrate 73 g, fibre 3.0 g, Ca 159 mg, Mg 170 mg, P 378 mg, Fe 5.8 mg, Zn 2 mg, thiamin 0.3 mg, riboflavin 0.2 mg, niacin 2.5 mg and ascorbic acid 88 mg (Protologue, 1918; Melak Haile Mengesha, 1965; Geremew Bultosa, 2007).

2.2.2 Origin and distribution

Tef [*Eragrostis tef* (Zucc.) Trotter] is an allotetraploid (2n = 4x = 40), indigenous cereal crop to Ethiopia. Within the genus *Eragrosti*s, 43 % of the species seem to have originated in Africa, 18 % in South America, 12 % in Asia, 10 % in Australia, 9 % in Central America, 6 % in North America and 2 % in Europe (Costanza, 1979). Of the 54 *Eragrostis* species listed in Ethiopia, 14 (26 %) are endemic (Cufodontis, 1974). The abundance of several species of *Eragrostis* in Ethiopia more than the other part of the world indicates tef originated and domesticated in Ethiopia (Vavilov, 1951). The exact date and location for the domestication of tef is still unresolved.

Grain cultivation of tef has been confined mainly to Ethiopia and to some extent the highlands of Eritrea. It is also grown in northern Kenya. Small-scale commercial tef production takes place in South Africa, the United States, Canada, Australia, Europe (Netherlands) and Yemen (Costanza *et al*., 1979). Tef is grown as a forage grass, for instance in South Africa, Morocco, Australia, India and Pakistan (Evert *et al*., 2008).

2.2.3 Domestication

Tef is perhaps descended from the closely related wild *Eragrostis pilosa* (L.) P. Beauv which is a tetraploid (2*n* =40), annual like tef and has a cosmopolitan distribution (Ingram and Doyle, 2003).

Previous studies have suggested 14 wild *Eragrostis* species as potential progenitors (Ingram and Doyle, 2003). According to these authors, the current consensus among the different studies on investigating the progenitor of tef indicated that *Eragrostis pilosa* is the most likely candidate wild progenitor of *Eragrostis tef*. *Eragrostis heteromera* is another previously proposed progenitor of tef genomes. Plastid DNA sequences from five varieties of tef and four *E. pilosa* accessions are identical and therefore are an informative with respect to the question of multiple origins of these polyploids (Ingram and Doyle, 2003). The *waxy* phylogeny also resolves the relationships among other allopolyploids, supporting a close relationship between the morphologically similar allotetraploids such as *E. macilenta*, *E. minor*, *E. mexicana*, *E. cilianensis* and other morphologically similar allopolyploids (Ingram and Doyle, 2003).

Eragrostis tef (Zucc.) Trotter is synonyms with *Poa abyssinica* Jacq.; *Eragrostis abyssinica* (Jacq.). In some Africans, tef is named as gewone bruin tef (ou bruin); Arabic: tahf; English: tef, teff, Williams love grass; Ethiopia: tafi (Oromo/Afar/Sodo), tafe-e (Had); t'ef, teff, taf (Amaringa and Tigringa languages); French: mil éthiopien; Malawi: chimanganga, ndzungula (Ch), chidzanjala (Lo) 23 (Teklu Yifru and Hailu Tefera, 2005).

2.2.4 Breeding objectives and methods

The first work on tef crossing was reported by Tareke Berhe (1975) and developed the method of crossing on tef. In the past, attempts to develop methods to improve breeding of tef cultivars have met with only limited success (Tareke Berhe and Miller, 1978; Tareke Berhe *et al*., 1989). However, recently, a highly client-oriented breeding applied to enhance the development and releasof a tef (*Eragrostis tef*) variety with farmer participation in Ethiopia. The main features include; cleaobjective, target cross, early-stage researcher selection, multi-location yield trial, farmer on-statioselection, judicious selection of few candidate varieties based on farmers' and researchers' selectionsfarmer managed on-farm trials, and release through the existing formal procedure (Getachew Belay et al., 2007). Biomass yield and kernel weight per main panicle were greater in newer cultivars, highly and linearly related to cultivar age, and positively and significantly correlated to grain yield. Number of spikelets per panicle is also greater in newer cultivars and significantly and positively correlated with grain yield. Improved plant height, panicle length, and kernels per panicle were a feature of most modern genotypes (Teklu Yifru and Hailu Tefera, 2005).

Tef improvement has relied mostly on mass selection from landraces for the development of new varieties (Yu *et al*., 2007). A hybrid between *E. tef* and *E. cilinensis* could set seeds, but the hybrid seeds fail to germinate (Tavassoli, 1986). This might be avoided through *in vitro* embryo rescue techniques. Likyelesh Gugsa *et al*. (1999) reported that 75 % of the cross of tef and *E. pilosa* (30-5) did not set seeds especially when the female was the wild spp. Hailu Tefera *et al*. (2003) have reported achievement of the interspecific cross between *E. tef*, cv. Kaye Murri and *E. pilosa* (30-5) when the wild was served as a donor (male). Moreover, two hundred recombinant inbred lines (RILs) derived from individual F_2 plants of the cross *E. tef* cv. Kaye Murri and *E. pilosa* (30-5) were developed using single seed descent method at the Debre Zeit Agricultural Research Center (DZARC), Ethiopia (Teklu Yifru and Hailu Tefera, 2005).

2.2.5 Cytogenetics

Meiosis in tef and its hybrids was difficult to observe due to few pollen mother cells in each anther. However, tef forms regular meiosis with 20 bivalents (Tavassoli, 1986). Flow cytometry research has shown that tef has a genome size of 730 Mbp, which is roughly the same size as diploid sorghum, and about 60 % larger than the diploid rice genome. It has also the smallest chromosomes reported among the Poaceae ranging from 0.8 to 2.9 μm, which has significantly hindered the cytogenetic research of this species (Yu *et al*., 2007).

Tef [*Eragrostis tef* (Zucc.) Trotter] is an allotetraploid with the chromosome set of 2n=4x=40. Most of the tef chromosomes are either metacentic or submetacentric, a few are acrocentric and one or two pairs are satellited. Twenty bivalents are normally formed during diakinasis to metaphase-I in pollen mother cells of tef varieties. Subsequent meiotic stages are also normal. Unlike some of its wild relatives, chromosomal races and aneuploidy are not reported in tef. However, molecular-cytogenetic techniques such as Fluorescence and Genomic *in situ* Hybridizations should be helpful for genome analysis in teff and related wild species (Likyelesh Gugsa *et al*., 2001).

2.2.6 Agronomy, production status and ecology

Planting of tef requires a firm moist seed bed to effect good soil moisture-seed contact due to the extremely small seed size. Tef has been harvested either as forage or seed production (Stallknecht *et al*., 1993). Tef seed can shatter if harvest is delayed. No specific fertility studies have been conducted, but rates similar to those suggested for millets or sorghums are recommended (Stallknecht *et al*., 1993).

Germination of tef normally takes place in 3-12 days after sowing. Tef germinates rapidly when planted at an average depth of 1.2 cm. In experiments, germination was above 90 % at temperatures of 15-35 °C; no germination occurred at 10 °C (Melak Haile Mengesha, 1965). However, Seyfu Ketema (1997) reported that germination proceeds within a temperature range of 10-27 °C.

A booting stage is not noticeable in tef: the inflorescences emerge suddenly from the upper leaf sheath without boot formation. The flowers open in the morning, 6:45-7:45 am, in response to light and temperature. Pollen is set free early in the morning (Stallknecht *et al*., 1993). In the inflorescence, floral maturity starts from the top and progresses downward, whereas in the spikelet it progresses from the base upward. Seeds mature within a month after pollination. The total growth cycle from sowing to maturity is 2-6 months (Evert *et al*., 2008).

In 2005/2006, 2006/2007 and 2007/2008 Ethiopian meher seasons, the area under tef production was estimated at 2, 246, 017; 2, 404, 674 and 2, 397, 121.45 ha with an average production of 0.969, 1.014 and 1.151 tonnes per hectare, respectively. It was the largest area covered compared to all cereals during the three consecutive years (CSA, 2008).

According to experiences gained so far from national yield trials conducted at different locations across the country, tef performs excellently at an altitude of 1800-2100 meters above sea level, annual rain fall of 750-850 mm, growing season rainfall of 450-550 mm (Seyfu Ketema, 1997).

2.2.7 Economic importance

Tef is the most preferred cereal by urban consumers as well as its producers and fetches the highest price than other cereals in Ethiopia. The reason for its preference include source of best quality in terms of color of injera, softness of the food and longevity in the shelef for human food; source of animal feed; tolerance to both high and low moisture stresses; high price for its grain and straw; low post harvest pest and disease problems; high longevity of the grain under farmer's traditional storage condition (Seyfu Ketema, 1997).

The principal use of tef grain for human food is the Ethiopian bread, injera. Injera is a major staple food, and provides approximately two-thirds of the diet in Ethiopia (Ingram and Doyle, 2003). 'Injera' is prepared in a range of sizes and is consumed with various sauces ('wot'), based on meat or pulses.

In Ethiopia, tef straw is used as forage, especially during the dry season. When it is mixed with clay it is used as plastering material for local houses and to make bricks, stoves, granaries, beds and pottery. Outside Ethiopia, tef is mainly grown for hay in South Africa and as green fodder in Morocco and India. In South Africa, it is planted for erosion control often in mixtures with *Eragrostis curvula* (Schrad.) Nees or other grass (Seyfu Ketema, 1993).

2.2.8 Constraints for production and research

Tedious crossing techniques, weak floral organ, lodging, drought, water logging, heat, and frost are production constraints of tef (Seyfu Ketma,1997; Likyelesh Gugsa, 2005). The seeds are so small that this alone makes the crop hard to deal with. Among the production constraints, lodging is a major bottleneck in tef production (Seyfu Ketema, 1997; Likyelesh Gugsa, 2005; Yu *et al*., 2007).

The positive correlation of lodging with yield and other important yield component traits indicate that improvement of lodging resistance in tef will be a challenging issue for a breeder (Yu *et al*., 2007). Likyelesh Gugsa (2005) and Yu *et al*. (2007) suggested that the problems of lodging will be achieved through wide hybridization with its wild relatives and /or with members of the *Pooideae* (wheat and barely) and *Oryzoideae* (rice). The process may be enhanced by tissue culture, embryo rescue.

Tef is relatively free of plant diseases when compared to other cereal crops. In Ethiopia, in locales where humidity is high, rusts and head smuts are important diseases. In Ethiopia, 22 fungi and 3 pathogenic nematodes have been identified on tef. Tef seedlings are also susceptible to Damping-off caused by *Drechslera poae* and *Helminthosporium poae* Shoemaker, when sown too early. Insect pests of tef in Ethiopia include Wello-bush cricket, *Decticoides brevipennis*, red tef worm, *Mentaxya ignicollis*, tef epilachna, and tef black beetle. Since tef has been limited to small areas in the United States, few disease and insect problems have been observed. However, a serious problem was observed in South Dakota where the stem boring wasp, *Eurytomocharis eragrostidis* (Howard) reduced forage yields by over 70 % (Endeshaw Bekele 1985; Seyfu Ketema, 1987).

2.2.9 Embryo rescue culture

Wide crosses in cereals may fail from two types of barriers such as prezygotic and postzygotic barriers. Prezygotic barriers may be caused due to intra and interspecific cross incompatibility as a result of disturbed pollen/stigma and pollen tube/style interactions. Postzygotic barriers may be caused due to sterility of the hybrid individual, death of a potentially viable embryo due to failure in endosperm development and selective chromosome elimination of one of the parental sets of chromosome during embryo development (Fernandes *et al*., 2000). Postzygotic barriers could be overcome by *in vitro* embryo rescue techniques. Interspecific crosses are often attempted to transfer desired traits such as tolerance to stress, high yield, high content of health-promoting compounds, or ornamental attractiveness from one into other important crop species (Korbin *et al*., 2008).

A method for the culture of barley (*Hordeum vulgare*) ovules isolated directly after fertilization is described and up to 28 % of these ovules contained a poorly developed embryo after 10-12 days of culture. After dissection, these embryos were cultured and about 20 % of them yielded mature plants (Töpfer and Steinbiss, 1985). Similarly, the attempts to rescue embryos after fertilization have been reported in inerspecific cross of tobacco [*Nicotiana repanda* (n=24)**N. tabacum* (n=24)] (Iwai *et al*., 1985), interspecific hybrids of *Gossypium* spp. (Stewart, 1981), interspecific hybrids of lily spp. (Chi, 2002) and interspecific hybrids of wheat (*Triticum aestivum*) (Sears and Deckard, 1982). The cross of wheat with its wild relative (Jonsson, 1995), interspecific crosses of tef (Likyelesh Gugsa *et al*., 1999) and cross of *Brassica napus* var. *oleifera* Metzger and wild mustard (*Sinapis arvensis* L.) (Lefol *et al*., 1999) were also rescued.

Likyelesh Gugsa (2005) reported that self fertilized ovaries and embryos of tef and its wild relatives were cultured after few days of self pollination to establish embryo rescue protocol for *Eragrostis* species such as *E. tef, E. mexicana*, and *E. paposa*. Embryos showed much higher (78-98 %) germination than ovaries (70-87 %) but ovaries significantly required shorter culture days than embryos. Besides one successfully rescued hybrid was achieved from the crosses of *E. tef* x *E. paposa* but it dies at seedling stage few days after transplanting, however, reason for the death was not reported. Therefore, successful protocol for routine achievement of hybrids from the wild crosses is important.

No hybrid was found among 2.9 million seeds produced by wild mustard grown in a garden in the presence of an herbicide-resistant transgenic cultivar. No more than six hybrids (0.0012 %) were obtained from 50,000 flowers of a male-sterile oilseed rape grown in the presence of wild mustard. Artificial hybrids grown in the presence of wild mustard, or hand-crossed, produced a few aborted seeds.

It may be concluded that a flower of these two species has a probability smaller than 10^{-10} of having an interspecific hybrid progeny. Artificial hybridization using *in vitro* ovary culture produced up to 1 seed per 100 pollinated flowers (0.01 %) (Lefol *et al*., 1999).

The present study aims to rescue F_1 hybrid of tef (*E. tef*) with wild relativew of *Eragrostis in vitro* in order to avoid the postzygotic barriers of the hybrids and get fertile seeds.

Somatic embryos in tef have been developed from roots and leaves (Endashaw Bekele, 1995), mature seeds (Kebebew Assefa *et al*., 1998) and immature embryos (Likyelesh Gugsa *et al*., 2006). However, somatic embryos from F_1 hybrids of domesticated tef varieties and tef with wild relatives of tef have not been achieved so far. Somatic embryos may be useful for micropropagation of F_1 hybird seeds and genetic transformation. Immature embryo cultures are reported in wheat and other cereals to be the very efficient explants for high regeneration capacity and genetic transformation (Liu and Li, 2007).

Somatic or asexual embryogenesis is the process by which somatic cells develop into plants (Vicient *et al*., 1998). Somatic embryos can be obtained via either direct or indirect somatic embryogenesis (Likyelesh, 2005; Hussien *et al*., 2006). In direct embryogenesis, the embryos originate directly from tissues in the absence of conspicuous callus proliferation (Hussien *et al*., 2006). Induction of somatic embryos directly from plant tissue is the most desirable approach in order to avoid somaclonal variation (Aoshima, 2005). Furthermore, it reduces the time required for plant regeneration, which may be beneficial to minimize culture-induced genetic changes (Fuentes *et al*., 2000). Likyelesh (2005) reported that direct somatic embryos were induced from pollinated pistilate and embro cultures of tef. In mature seed cultures of tef, the frequency of somatic embryogenesis was in the range of 0-68.3% (Kebebew *et al*., 1998).

Gene transfer methods are an essential part of the new technologies that are altering conventional plant breeding and have become indispensable tools. However, in some species the lack of an efficient regeneration method is a great bottleneck in the use of transformation technology. The development of regeneration methods based on somatic embryogenesis could be the solution (Vicient and Martínez 1998).

2.2.9.1 Factors that affect embryo rescue

Factors that affect *in vitro* culture in tef are explant types, media compositions, genotypes, stages of develpoment of the donor plants, the parental forms used for hybridization and culture conditions (Kebebew Assefa *et al*., 1998; Likyelesh Gugsa, 2005; Korbin *et al*., 2008).

The media that have been used so far for embryo rescue are MS (Murashige and Skoog, 1962), B_5 (Gamborge *et al*., 1968), NN (Nitsch and Nitsch, 1969) and K99 (Kumlehn, 99 unpublished). The vitamins and hormones are similar to ovary cultures of wheat.

Plantlets regenerate on half MS medium without growth regulators from somatic embryogenesis of saffron in three months (Shiebani *et al*., 2007). Growth of somatic embryos were in MS+NPGR+6% sucrose. Matured somatic embryos were transferred to half MS medium without growth regulators. Regeneration of plantlet from capitulum explants of gerbera was in half MS medium (Kanwar and Kumar, 2008). Somatic embryogenesis was better achieved in auxins (2,4-D) containing medium and the highest SE formation for varieties DZ-01-196 and DZ-Cr-37 were achieved when 2.2-3.3 mg/l 2, 4-D were used. The author also described that subculturing was essential to increase productivity of somatic embryos and thus, separation of somatic embryos into small pieces and subsequent culture in fresh media increased the number of plantlets per somatic embryo.

Depending on genotypes, embryogenic calli, which were transferred to MS shoot induction medium showed 0-25% regeneration rate (Likyelesh, 2005) and 96 % of the F1 hybrid embryos of *Triticum aestivum*Lymus mollis* have developed into hybrid plants (Jonsson, 1995). The capacity of somatic embryos to regenerate shoot and root and the high number of plantlets per explant was depended on the genotypes, culture media composition and the size of the embryo (Likyelesh, 2005).

2.2.10.2 Applications of embryo culture

Embryo rescue is essential to overcome embryo inviability and seed dormancy. It is also useful for germination of seeds of obligatory parasite without the presence of host, monoploid production, prevention of embryo abortion with early ripening stone fruits, F_1 hybrid seed propagation, vegetative propagation, and tools for research in experimental embryogenesis (Taji, 2003). The rapid improvement in somatic embryogenesis methods allow the use of somatic embryos in plant micropropagation as synthetic seeds. However, practical applications of somatic embryogenesis are not limited to synthetic seed technology. Somatic embryogenesis can be used in the regeneration of genetically transformed plants, polyploid plants, or somatic hybrids. Moreover, promising results indicate the possibility to use somatic embryogenesis in cell selection programs and germplasm cryopreservation. The application of somatic embryogenesis to plant virus elimination, metabolite production, and in vitro mychorrhizal initiation has been investigated (Vicient and Martínez, 1998).

3. OBJECTIVES OF THE STUDY

3.1 General objective

Regeneration of plants from gynogenic cultures of wheat (*T. durum* and *T. aestivum*) and *in vitro* rescue of immature embryos of the F_1 hybrids of the interspecific crosses of *Eragrostis tef* with its wild relatives.

3.2 Specific objectives

3.2.1 Specific objectives for unpollinated ovary cultures of wheat

- To investigate the effect of explant types on induction of embryonic tissues.
- To investigate the effect of light and dark on induction of embryonic tissues.
- To determine the developmental stages of unpollinated ovary donor plants for induction of embryonic tissues.
- To optimize cold pretreatment for induction of embryonic tissues.
- To optimize the type and concentration of sugar for induction of embryonic tissues.
- To investigate the effect of different media on induction of embryonic tissues.
- To investigate the effect of plant growth regulators on induction of embryonic tissues.
- To investigate the effect of seasons on induction of embryonic tissues.
- To investigate the effect of plant growth regulators on regeneration of shoots.
- To investigate the effect of genotypes on induction of embryonic tissues and regeneration of plantlets.
- To regenerate haploid plants from unpollinated ovary cultures of wheat (*Triticum* spp.).

3.2.2 Specific objectives for embryo rescue cultures of tef

- To regenerate F_1 hybrid plants from interspecific crosses of *Eragrostis tef* with its wild relatives.
- To optimize the developmental stages of interspecific crosses of *Eragrostis tef* with its wild relatives for the induction of somatic embryos.

- To optimize the concentrations of 2,4-D to induce somatic embryos from interspecific crosses of *Eragrostis tef* with its wild relatives.

4. MATERIALS AND METHODS

4.1 UNPOLLINATED OVARY CULTURES OF WHEAT

4.1.1 Plant materials used

Two varieties of bread wheat (*Triticum aestivum*), Simba and Galema and two varieties of durum wheat (*Triticum turgidum*), Ude and Yerer were used in this study. Seeds of these varieties were obtained from Debre Zeit Agricultural Research Center (DZARC) and Holeta Agricultural Research Center (HARC). Ovary donor plants of each variety were sown in 30 cm diameter pots filled with black soil and kept in a glasshouse at DZARC. Two pots were assigned for each variety. In each pot, 5 seeds were sown in two weeks interval. All varieties were continously grown for one year.

4.1.2 Collection of tillers and surface sterilization

Immature spikes were collected when the owns first emerged from the flag leaf. Tillers from the second nodes were collected using sterile scissors, placed in a sterile beaker containing 300 ml of distilled water till it covers the stem, labeled and kept at 4 ^{o}C for 1-25 days. After cold pretreatment, spikes were sterilized with 70 % ethyl alcohol for 1 min, followed by 20 % sodium hypochlorite of the stock 5.25 % w/v and four drops of Tween 20 for 10 min, and then rinsed with sterile double distilled water four times.

4.1.3 The effect of explant types on induction of embryonic tissues

During preliminary experiments, three types of explants (spike, spikelet and ovary) were taken from different stages of development of the four varieties and cultured in MS medium supplemented with 1 mg/l 2,4-D. They were pretreated at 4 ^{o}C (1-25 days) and kept both in dark and light culture conditions. Embryonic tissues were transferred into 25 ml shoot regeneration medium (MS) supplemented with combinations of 1 mg/l 2,4-D with 0.5-2 mg/l BAP or 1-2 mg/l BAP with 1 mg/l GA_3.

4.1.4 Ovary excision and plating

Following surface sterilization, the ovaries were excised using scissors, forceps, scalpel blades and needles aseptically under stereo microscope. Twenty ovaries were placed in each Petri-dish (15x100 mm) containing 20 ml induction medium supplemented with different concentrations and combinations of auxins and cytokinins, sealed with parafilm tightly under aseptic conditions and kept in the growthroom under both dark and light culture conditions.

4.1.5 The effect of light and dark on induction of embryonic tissues

Pretreated and non pretreated ovaries of all varieties were cultured into two culture conditions, dark and light, using MS medium supplemented with 0.5 mg/l of 2,4-D.

4.1.6 Identifying the appropriate harvesting stages of unpollinated ovary donor plants for induction of embryonic tissues

The morphology of wheat spikes was taken in order to determine the appropriate harvesting stage of unpollinated ovary donor plants for maximum response of embryonic tissues. Spikes taken from variety Simba were categorized into three stages and unpollinated ovaries were excised. These were before the emergence of own from the boot (stage I), spike length of 5-9 cm which might be equivalent to uninucleate stage of wheat anther; when the owns emerged out from the boot, stage II (spike length of 10-14 cm) which might be equivalent to late uninucleate to binucleate stage of wheat anther; when the owns completely emerged out from the boot, stage III (spike length of 15-18 cm) which might be equivalent to the trinucleate stage of wheat anther. Ovaries from all stages were kept under light culture condition.

4.1.7 The effect of cold pretreatment durations on induction of embryonic tissues

Spikes at stage II of the four varieties were cold pretreated at 4 ^{o}C for variable durations (5, 10, 15, 20 and 25 days). Because stage II was determined as best stage through series of experiments and used for the remaining experiments. Ovaries were plated in a disposable Petri-dish containing 20 ml MS medium supplemented with 1 mg/l of 2,4-D. To confirm the effect of cold pretreatment, ovaries from spikes of all varieties without cold pretreatment were cultured in the same way. All culture Petri-dishes were kept under light culture condition.

4.1.8 The effect of sucrose and maltose on induction of embryonic tissues

Spikes at stage II of variety Simba were collected and cold pretreated (4 ^{o}C) for 15 days. They were used to examine the effect of sucrose and maltose on induction of embryonic tissues at 30, 60, or 90 g/l concentrations. The ovaries were plated in a Petri-dish containing MS medium supplemented with 1 mg/l of 2,4-D under light culture condition.

4.1.9 The effect of culture media on induction of embryonic tissues

Three types of media MS (Murashige and Skoog, 1962), N_6 (Chu, 1978) and B_5 (Gamborg *et al.*, 1968) were used as induction media. Each type of medium was supplemented with 1 mg/l 2,4-D and 30 g/l maltose. The best medium for induction of embryonic tissues was investigated using variety Simba.

4.1.10 The effect of different concentrations and combinations of PGRs on induction of embryonic tissues

Ovaries from stage II of the four varieties were cold pretreated at 4 ^{o}C and cultured in Petri-dishes containing MS medium supplemented with twelve different concentrations and combinations of 2,4-D and KIN and 30 g/l maltose. All Petri-dishes were kept under light culture condition.

In addition to 2,4-D and KIN combinations, ovaries of varieties Ude and Galema were also cultured in Petri-dishes containing MS medium supplemented with four different combinations of 2,4-D and BAP and 30 g/l maltose.

4.1.11 The effect of embryo promoting medium on induction of embryonic tissues

Enlarged ovaries of three varieties, Simba, Galema and Yerer were transferred into MS medium supplemented with three different concentrations of BAP and 30 g/l maltose. They were named as embryo promoting medium. They were EP_A, MS with 2.5 mg/l BAP; EP_B, MS with 5 mg/l BAP; and EP_C, MS with 10 mg/l BAP.

4.1.12 The effect of seasons on induction of embryonic tissues

Ovaries of the four varieties were cultured in three seasons namely January-March, April-June and July-September to investigate whether difference exists among varieties and seasons on induction of embryonic tissues. Ovaries were cultured on MS medium supplemented with 1 mg/l of 2,4-D and 30 g/l maltose.

4.1.13 The effect 2,4-D, NAA, IAA, KIN and BAP on regeneration of shoots

After the observations of preliminary results of shoot regeneration, an experiment was conducted to test the effect of different concentrations and combinations of kinetin with 2,4-D or NAA for shoot regeneration. Embryonic tissues of all varieties, that were derived from MS or N_6 medium, were transferred into MS regeneration medium supplemented with five combinations of 2,4-D and KIN, three combinations of NAA and KIN, one combination of 2,4-D and BAP, one combination of IAA and BAP and one treatment without PGR. All treatments were supplemented with 60 g/l sucrose. All cultures were incubated in the growthroom under light culture condition.

4.1.14 The effect of different concentrations of GA_3 on elongation of shoots

Embryonic shoots of varieties of Yerer and Simba were cultured on MS medium supplemented with 60 g/l of sucrose and different concentrations of GA_3 (1, and 1.5 mg/l).

4.1.15 Culture conditions

Most of the Petri-dishes were kept in growthroom incubated under 16 h light (1032-1557 lux flourescent intensity) and 8 h dark cycles. The temperature was adjusted at 24 ± 4 ^{o}C. Sometimes, it was increased above this temperature when light was off. The temperature of the dark culture condition was adjusted at 26 ^{o}C.

4.1.16 Acclimatization and growing of plantlets in the glasshouse

Plantlets of varietiesYerer having shoot length of 2-14 cm and root length of 4-10 cm and Simba having shoot length of 2 cm and root length of 6 cm were removed from Majenta jar using forceps. The roots were washed with distilled water to remove the gelrite and placed into pots filled with 3:2:1 ratios of black soil, compost and sand, respectively. Each pot was covered with plastic bag for one week and placed in the growthroom. Due to high humidity in the room, the plastic bags were removed after one week. After two weeks, pots were transferred into glasshouse.

4.2 EMBRYO RESCUE CULTURES OF TEF

4.2.1 Plant materials used

The seeds of two wild species of *Eragrostis*: *Eragrostis pilosa* (30-5), *Eragrostis pilosa* (37 80 82) and *Eragrostis curvula* and four varieties of tef: *Eragrostis tef* cv. Kaye Murri, *Eragrostis tef* cv. DZ-Cr-387, *Eragrostis tef* cv. DZ-Cr-37 and *Eragrostis tef* cv. DZ-01-196 were used in this study. They were obtained from tef sections of DZARC and the seeds were sown in Petri-dishes until they were germinated. After 8-10 days, they were transplanted and grown in small sized pots of 11-13 cm diameter containing light soils in the glasshouse as described for unpollinated ovary cultures of wheat. *E. pilosa* (30-5) and *E. pilosa* (37 80 87) were sown one week later and that of *E. curvula* was sown a week earlier than the sowing date of tef varieties.

4.2.2 Interspecific crossing of *Eragrostis tef* with its wild relatives

At the heading stage, the anthers of wild varieties were emasculated in the afternoon from 3.00-4.00 pm under stereo microscope using very fine forceps and needles. To avoid confusion of identifying pollinated and unpollinated florets of the mother plants, all the florets around the emasculated florets were removed. The wild and domesticated varieties were used as a female and male plants respecively. The domesticated varieties were kept in refrigerator overnight. The next day, from 6.00-9.00 am, stamens were excised from the male plant and the pollen was transferred to the female plant. Only eight cross combinations were carried out (Table 3). Pollinated florets from eight cross combinations were excised after 3-12 days of artificial crossing and cultured on induction medium supplemened with different concentrations of 2,4-D.

Table 3. Interspecific crosses between tef varieties and wild relatives.

	Male plants (domesticated varieties)			
Female plants (Wild relatives)	DZ-Cr-37	DZ-Cr-387	DZ-01-196	Kaye Murri
E. pilosa (30-5)	✓	✓	✓	✓
E. pilosa (37 80 87)	✓	✓	**X**	✓
E. curvula	**X**	✓	**X**	**X**

✓ **Sign indicated crossing was done between the crossponding male and female parents.**

X indicated crossing was not done between the corresponding parents.

4.2.3 Collection of panicles and surface sterilization

After 3-12 days of artificial crossing, panicles were removed from the plants and placed in a beaker containing distilled water and taken immediately to laminar hood cabinet. Following collection of panicles, the spikelets were cut using sterilized scissors, scalpel and forceps. They were placed in a small Petri-dish containing 4 % sodium hypochlorite of the original 5.25 % w/v for eight minutes and rinsed four times with double distilled water for surface sterilization.

4.2.4 Excision of florets, pollinated ovaries and somatic embryos

Following surface sterilization, the florets from sterilized spikelets were detached and cultured in Petri-dishes containing MS medium supplemented with 0, 0.1, 0.5, 1, 1.5, and 2 mg/l 2,4-D, sealed with parafilm and kept in light culture conditions as described in culture condition of unpollinated ovary cultures of wheat. During the first subculturing, ovaries were excised from the cultured florets and transferred into the same induction medium. Somatic embryos were excised under stereo microscope from the cultured pollinated ovaries and they were transferred into regeneration medium.

4.2.5 Determination of the appropriate harvesting stages of panicles for induction of somatic embryos

F_1 hybrids of *E. pilosa* (30-5)**E. tef* cv. Kaye Murri were used to determine appropriate harvesting stages of panicles for induction of better percentage of somatic embryos. After artificial crossing, panicles were collected at different days (3, 6, 9 and 12). Their florets were excised and placed in Petri-dishes containing 20 ml MS medium supplemented with 1 mg/l of 2,4-D and 30 g/l maltose. The dishes were placed under light culture condition which was similar to the culture condition of unpollinated ovary cultures of wheat.

4.2.6 The effect of different concentrations of 2,4-D on induction of somatic embryos

After 5-6 days of artificial crossing, pollinated florets of F_1 hybrids of tef with its wild relatives were cultured in MS medium supplemented with 0, 0.1, 0.5, 1, 1.5 or 2 mg/l of 2,4-D and 30 g/l of maltose. They were kept in light culture condition. After 5-6 weeks from the initial culture, somatic embryos were transferred into Petri-dishes containing 20 ml of regeneration medium. The regeneration medium was half strengthen MS medium without PGRs. The first subculturing of the embryos was done in the same dish containing the same amount of the regeneration medium. The second subculturing of the embryos

was done in test tubes contanining 15 ml of the regeneration medium. Finally, they were subcultured in Majenta jars containing 50 ml of the regeneration medium.

4.2.7 Acclimatization and growing of plantlets in the glasshouse

Acclimatization and growth conditions of the F_1 hybrids of tef plantlets were similar to unpollinated ovary cultures of wheat plantlets. However, the plantlets of F_1 hybrids of tef took longer time (12 weeks) to be transferred into pots. Morphological characteristics such as plant height, panicle length, culm length and peduncle length of rescued hybrids of the main tiller were measured and the total number of tillers were counted from each pot which contained a single plant.

4.3 Preparation of stock solutions and media

Stock solutions were prepared for all the media used in this experiment. The stock solutions of macronutrients and micronutrients were concentrated 10x and 100x, respectively. Vitamins were concentrated 1000x and 100 mg/l of myoinositol was added during media preparation. Growth regulators of 50 mg were dissolved in 2.5 ml of ethanol or sodium hydroxide before water was added. For 50 mg of each growth regulator type, 50 ml of water was added and stirred until a clear homogenous solution was formed. They were stored in separate glass bottles. The stock solutions were kept at -20 ^{o}C.

The components of the media were macronutrients, micronutrients, vitamins, sugar and hormones. The media were solidified with 2 g/l of gelrite and pH was adjusted at 5.8 using sodium hydroxide and hydrochloric acid. They were autoclaved at a temperature of 121 ^{o}C and a pressure of 100 kpa for 15 minutes. However, hormones were filter sterilized and added in the media after autoclaving. The media were stored at a temperature of 4 ^{o}C.

4.4 Data collections and analysis

Each independent experiment had 1-3 replications and repeated 1-4 times. In MS induction medium, 20 ovaries and in MS regeneration medium, 3 embryonic tissues were used as a unit of replication. For embryo promoting medium, three-five enlarged ovaries were used as a unit of replication. For embryo rescue cultures, 5 pollinated florets and 2-3 somatic embryos were used as a unit of replication in induction and regeneration medium, respectively. Subculturing was carried out every 2-3 weeks. The response of ovaries on direct embryogenesis was checked every 3-4 days. Contaminated Petri-dishes were discarded immediately and the number of embryonic tissues per Petri-dish were counted. The frequency of responsive ovaries was assessed in terms of percentage of developed ovaries and induced

embryonic tissues. Moreover, the response of cultured florets were checked every week and the frequency of induced somatic embryos were recorded per dish for each treatment.

All the experiments were carried out in Complete Random Design (CRD). The analysis of variance (ANOVA) was conducted using SAS computer software (Version, 1999). The possible pairs of treatment means were compared using LSD/Duncan test. If $P \leq 0.05$, there is significant difference among the treatments.

5. RESULTS

5.1 UNPOLLINATED OVARY CULTURES OF WHEAT

5.1.1 The effect of explant types on the percentage of enlarged ovaries and induced embryonic tissues

The effect of explant types on the percentage of enlarged ovaries and induced embryonic tissues was presented in Table 4 and Figs 1 and 2. These results indicated that percentage of enlarged ovaries was maximum for ovary cultures of variety Galema (90.8 %) followed by spikelet cultures of variety Simba (83.3 %) and ovary cultures of variety Ude (80.0 %). With the exception of variety Simba, ovary cultures of the three varieties responded better in percentage of enlarged ovaries. However, spike cultures of all varieties responded least. In all types of explant cultures (spike, spikelet and ovary), none of the varieties induced embryonic tissues and shoots except ovary cultures of variety Simba. Ovaries enlarged after 7-10 weeks from spike and spikelet cultures. However, enlargement of ovaries started after 10 days for direct ovary cultures. Thus, the response of spike and spikelet cultures were very late. All ovaries cultured under light culture condition were green and white for those ovaries cultured under dark (Fig 5a, b, c, d, e, f, g and h). The size of enlarged ovaries were largest for variety Galema and least for variety Yerer (Appendix 6). Unlike direct ovary cultures, spike and spikelet cultures were lately responsive and did not induce embryonic tissues and shoots. Thus, ovary cultures were selected for further experimentations.

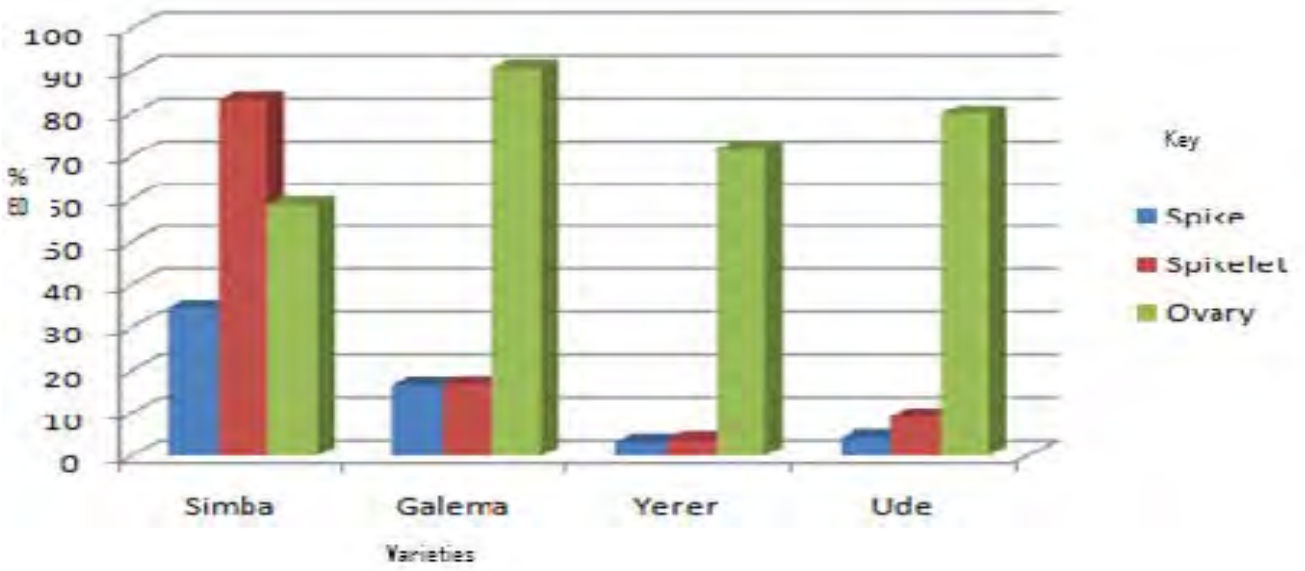

Fig 1. The effect of explant types on the percentage of enlarged ovaries using four varieties of wheat.

Table 4. The effect of explant types on the percentage of enlarged ovaries, embryonic tissues and number of shoots regenerated.

Varieties	Types of explants											
	Spike				Spike let				Ovary			
	NCE	%EO±SD	%ET±SD	NSR	NCE	%EO±SD	%ET±SD	NSR	NCE	%EO±SD	%ET±SD	NSR
Simba	56	34.5±13.3	0.0±0.0	0	120	83.3±6.4	0.0±0.0	0	75	58.7±14.2	1.3±0.0	1
Galema	30	16.4±10.3	0.0±0.0	0	200	16.8±3.2	0.0±0.0	0	120	90.8±7.8	0.0±0.0	0
Yerer	18	3.2±0.0	0.0±0.0	0	40	3.8±2.3	0.0±0.0	0	60	71.6±8.7	0.0±0.0	0
Ude	39	4.3±2.3	0.0±0.0	0	60	9.2±1.4	0.0±0.0	0	60	80.0±9.6	0.0±0.0	0

NCE=Number of cultured explants, EO=enlarged ovaries, ET=Embryonic tissues, SD=Standard deviation, NSR=Number of shoot regenerated.

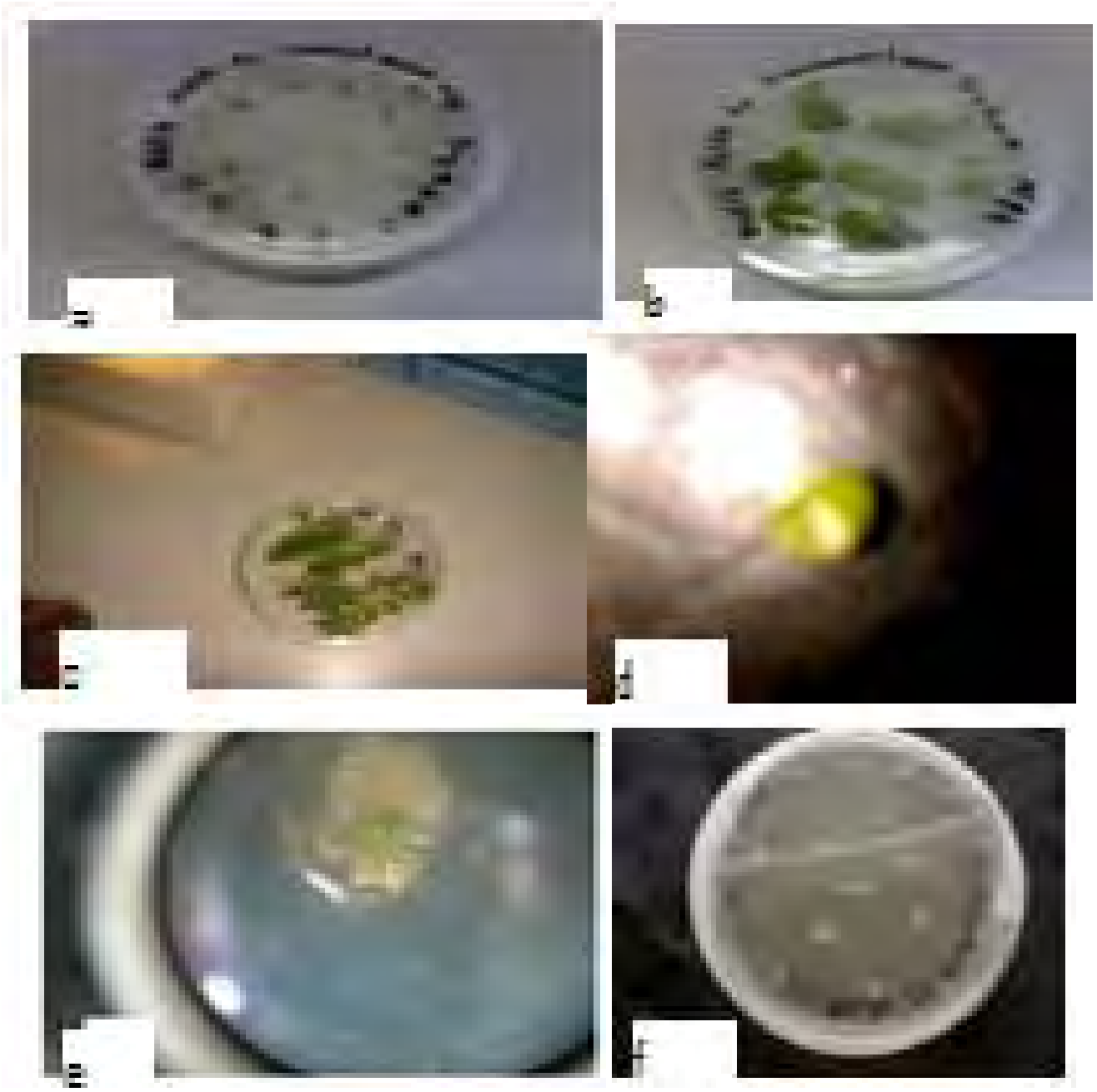

Fig 2. Different types of explants of variety Simba and their sequential development. a) Ovary culture, b) Spikelet culture, c) Spike culture, d) Enlarged ovaries, e) Embryonic tissue, f) Enlarged ovaries dried, collapsed and became white after 4 weeks if they did not induce embryonic tissues.

5.1.2 The effect of light and dark on enlargement of ovaries

The effect of light and dark culture conditions on the percentage of enlarged ovaries was presented in Figs 3, 4 and Appendix 1. Under the same culture condition, variety Ude gave maximum percentage of enlarged ovaries (89.3 %) followed by variety Simba (89.0 %) (Appendix 1). Moreover, under the same culture condition, variety Galema gave maximum percentage of enlarged ovaries (86.7 %) followed by variety Ude (82.5 %) (Appendix 1). Ovaries of all varieties showed enlargement after 10 days starting from the first day of culturing (Fig 4).

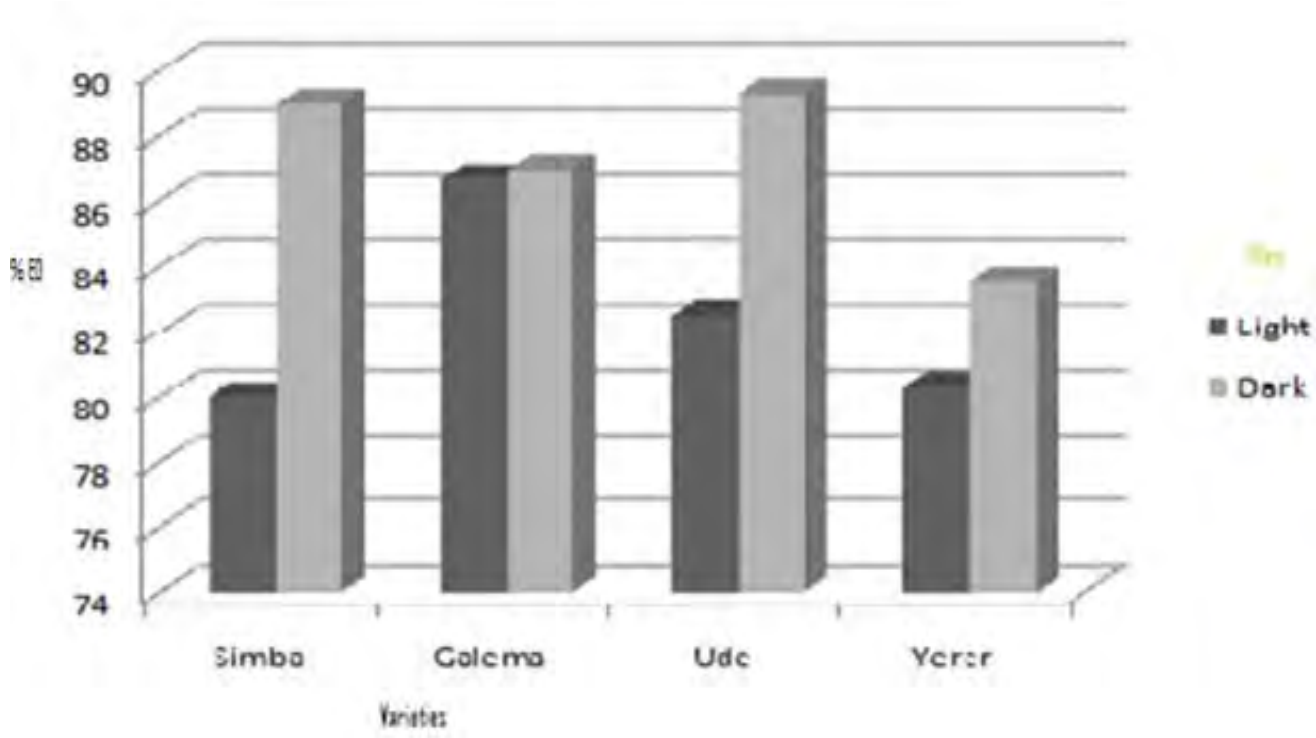

Fig 3. The effect of light and dark on enlargement of ovaries (%) using all varieties.

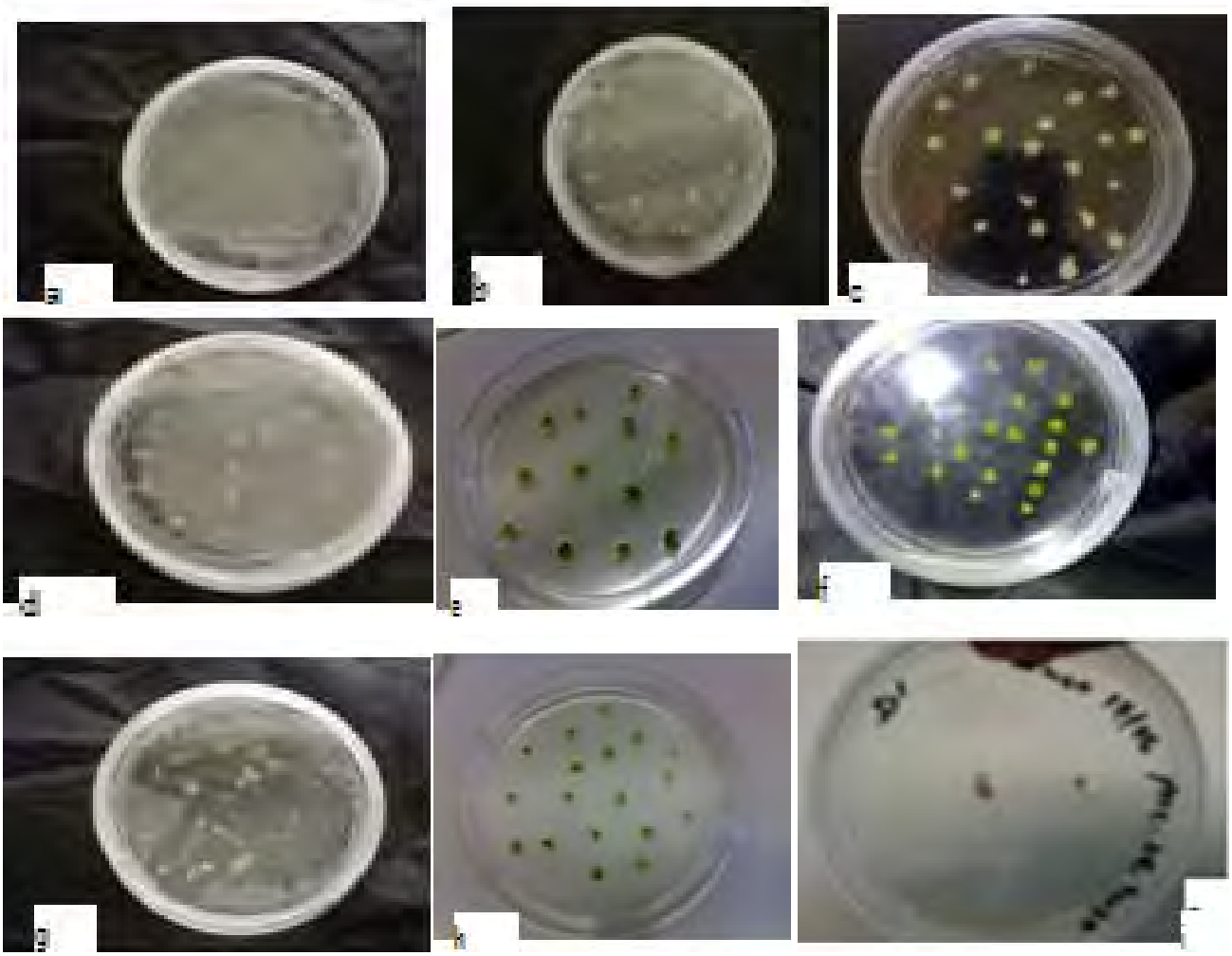

Fig 4. Ovary cultures of the four varieties under light and dark culture conditions. a) Ude under dark, b) Simba under dark, c) Galema under dark, d) Yerer under dark, e) Galema under light, f) Ude under light, g) Yerer under light, h) Simba under light, i) embryonic tissues of variety Simba.

5.1.3 The effect of light and dark on induction of embryonic tissues

Under dark culture condition, none of the cultured ovaries of varieties Simba, Galema and Yerer induced embryonic tissues (Fig 5 and Appendix 1). However, variety Ude induced embryonic tissues (1.6 %) that could not be differentiated into shoots (Fig 5 and Appendix 1). Under light culture condition, all varieties induced embryonic tissues. For all varieties, light culture was selected as the better culture condition on induction of embryonic tissues and used for further experiments.

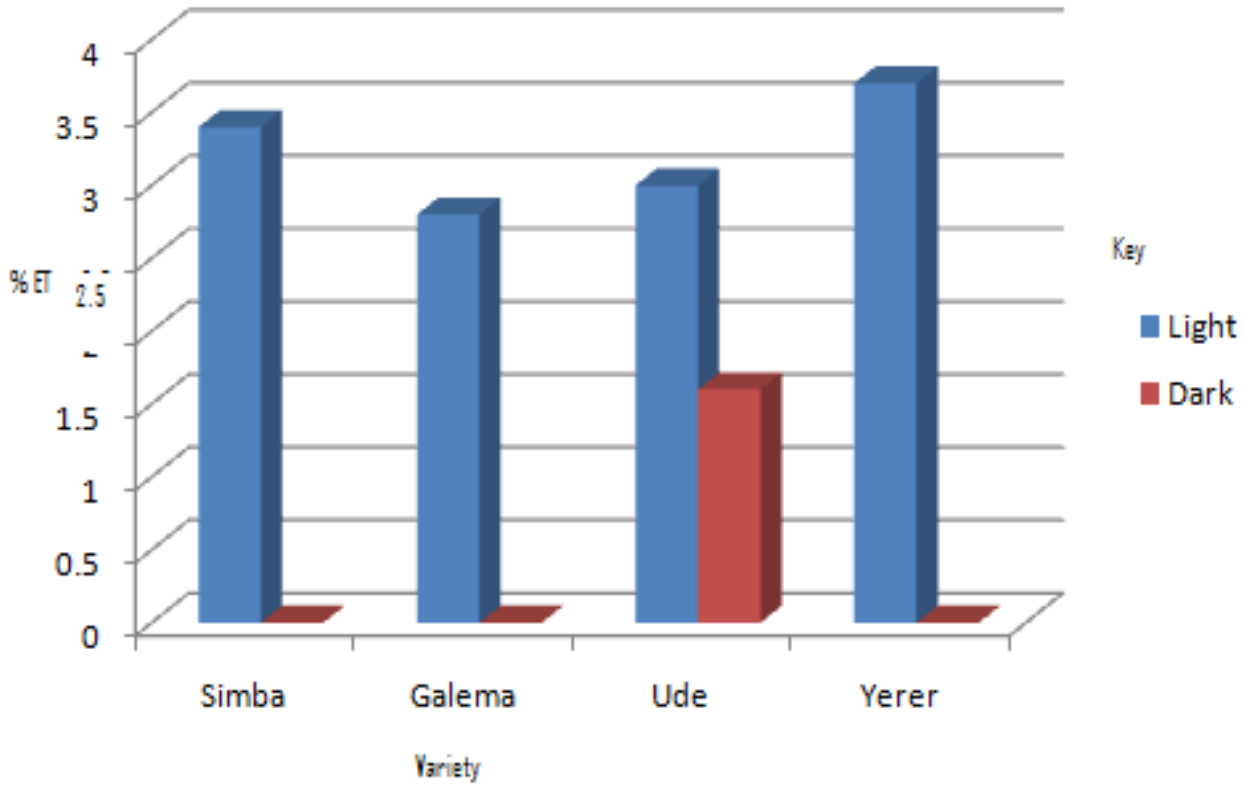

Fig 5. The effect of light and dark on induction of embryonic tissues.

5.1.4 Determination of appropriate harvesting stages of spikes

Ovaries taken from stage II induced maximum percentage of embryonic tissues (25 %) followed by stage I (11.6 %) (Table 5). Better number of embryonic tissues per Petri-dish were induced compared to ovary cultures from other stages (Fig 6). Highly significant difference ($P \leq 0.01$) was observed among the stages (Table 6). The size of ovaries at the time of culturing was increased as the stage of the donor plants got older and older. Although the size of enlarged ovaries for stage III was the largest, they could not sprouted out to give amenable amount of embryonic tissues and were found to be the least responsive (5.0 %) (Table 5). Thus, stage II was determined as optimum stage for induction of direct embryogenesis and used for other experiments.

Table 5. Determination of appropriate harvesting stages of spikes for induction of embryonic tissues using variety Simba.

Stages of spike	Stage I	Stage II	Stage III
Cultured ovaries	120	120	120
ET	14	30	6
%ET±SD	11.6±4.7	25.0±9.6	5.0±2.9

ET=Embryonic tissues, SD=Standard Deviation.

Table 6. ANOVA for determination of the best responsive stage of ovaries for induction of embryonic tissues.

S of V	DF	MS	F value	p
Stage	2	49.8	28.0	0.0044**
Rep	2	0.8	0.4	0.67
Error	4	3.8		
Total	8	54.4		

S of V=Source of variation= very significant, p≤0.01.**

Fig 6. Induced embryonic tissues from cultured ovaries of variety Simba, stage II.

5.1.5 The effect of cold pretreatment durations on induction of embryonic tissues

Ovaries cultured at 15 d cold pretreatment induced maximum percentage of embryonic tissues (8.3-20.0 %) for all varieties followed by 10 d (5.8-10.8 %) (Table 7 and Appendix 2). It was significantly different from all pretreatment durations (Table 7). The control responded least percentage of embryonic tissues (3.3-8.3 %) following 25 d pretreatment (0.0-5.8 %) for all varieties and 20 d pretreatment (1.7-4.2 %) for all varieties except variety Simba. There was no significant difference among the control, 25 d and 20 d pretreatments (Table 7). Compared to other varieties,variety Simba induced maximum number of embryonic tissues in most of the pretreatment conditions (Table 7). It was significantly different from varieties Ude and Galema. It was not significantly different from variety Yerer. Varieties Ude and Galema induced relatively least percentage of embryonic tissues in most of the pretreatment conditions. There was no significant difference between them. Therefore, 15 d cold pretreatment was taken as the optimum for induction of embryonic tissues and used for the remaining experiments.

Table 7. Effect of cold pretreatment durations on the percentage of embryonic tissues. It is shown as mean of % ET±SD.

Percentage of embryonic tissues							
Varieties	**Pretreatments**						**Mean of the means**
	0 d	5 d	10 d	15 d	20 d	25 d	
Simba	6.0±3.5	8.3±2.4	10.0±4.0	20.0±4.0	9.2±1.9	5.8±1.9	**9.2a**
Yerer	8.3±4.4	9.2±4.2	10.8±2.5	16.6±4.5	3.3±2.4	0.8±2.5	**8.6a**
Ude	5.0±2.9	5.8±2.4	5.8±2.4	8.3±2.4	4.2±2.5	1.6±2.4	**5.6b**
Galema	3.3±2.4	6.0±2.4	6.0±2.4	13.3±2.4	1.7±3.2	0.0±0.0	**4.7b**
Mean of the means	**4.2c**	**7.9b**	**8.8b**	**14.6a**	**4.6c**	**2.9c**	

ANOVA for the effect of cold pretreatment on % of induced ET

Source of variation	DF	MS	F-value	P
Variety	3	87.4	6.6	0.0006**
Pretreatment	5	192.0	14.5	<0.0001**
Rep	2	1.4	0.1	0.9006
Error	61	13.2		
Total	71	294.0		

Means with the same letter along the row and column are not significantly different at p≥0.05 using Duncan multiple range test. ** indicates significant difference at p ≤0.1.

5.1.6 The effect of sucrose and maltose on induction of embryonic tissues

Ovaries cultured in MS medium supplemented with 30 g/l of maltose gave maximum percentage of embryonic tissues(12.5 %) followed by 30 g/l sucrose and 60 g/l maltose (Table 8). Those ovaries cultured in a medium supplemented with 60 g/l sucrose or 90 g/l of maltose and sucrose did not induce embryonic tissues. However, 90 g/l of sucrose and maltose gave large sized ovaries compared to other concentrations of maltose and sucrose (data not shown). According to this specific investigation, 30 g/l of maltose was taken as the optimum type of sugar used to induce maximum number of embryonic tissues from ovary cultures. It was used for other induction experiments.

Table 8. The effect of sucrose and maltose on induction of embryonic tissues using variety Simba.

Concentrations of sucrose and maltose (g/l)						
Types of sugar	30		60		90	
	Cultured ovaries	%ET±SD	Cultured ovaries	%ET±SD	Cultured ovaries	%ET±SD
Maltose	40	12.5±2.5	60	3.3±2.3	40	0.0±0.0
Sucrose	40	5.0±3.5	40	0.0±0.0	40	0.0±0.0

5.1.7 The effect of culture media on the percentage of induced embryonic tissues using variety Simba

The effect of different media on the percentage of induced embryonic tissues of variety Simba indicated that there was significant difference ($P \leq 0.05$) among media on the percentage of induced embryonic tissues (Table 9). The enlarged ovaries in N_6 and MS media were larger in size and looked green pots. However, the enlarged ovaries in B_5 were pale green and their sizes were relatively smaller than the two media (Appendix 7). A total of 60, 40 and 40 ovaries were cultured in MS, N_6 and B_5 media, respectively. The percentage of induced embryonic tissues were better in MS medium (20.0 %) followed by N_6 medium (5.0 %). In B_5 medium, none of the enlarged ovaries induced embryonic tissues (0.0 %).

Table 9. Analysis of variance for the effect of different media on the induction of embryonic tissues from ovary cultures of variety Simba.

		% ET		
Source of variation	**DF**	**MS**	**F-value**	**p**
Media	2	304.2	24.3	0.04*
Rep	2	18.8	1.5	0.4
Error	2	12.5		
Total	6	335.5		

***=significant at p≤0.05**

5.1.8 The effect of different growth regulators on direct embryogenesis

Maximum percentage of embryonic tissues were induced in MS medium supplemented with 1 mg/l of 2,4-D combined with 1 mg/l of KIN for all varieties except variety Galema (Table 10). In this treatment, the highest (35 %) and the second highest (26.6 %) percentage of embryonic tissues were obtained from varieties Yerer and Simba, respectively (Table 10). In the same treatment, varieties Ude and Galema induced 13.3 % and 8.3 % embryonic tissues respectively. Varieties Simba and Yerer offered the second and third highest percentage of embryonic tissues in 1 mg/l of 2,4-D and in the combination of 1 mg/l 2,4-D with 2 mg/l of KIN. Variety Galema induced the first, second and third highest percentage of embryonic tissues in 1 mg/l 2,4-D, 1 mg/l of each of 2,4-D and KIN and in 2 mg/l of 2,4-D respectively. Variety Ude induced the second highest percentage of embryonic tissues in 1 mg/l 2,4-D combined with 2 mg/l of KIN. In most of the treatments, varieties Simba and Yerer induced better percentage of embryonic tissues than varieties Ude and Galema (Table 10). These varieties gave high quality embryonic tissues that can easily differentiated into shoots (Fig 7). The least amount of embryonic tissues (3.3 %) were obtained from variety Yerer in 2 mg/l 2,4-D combined with 2 mg/l KIN. In variety Simba, the percentage of embryonic tissues increased in larger amount in different levels of 2,4-D than in the same level of 2,4-D at different levels of KIN. Unlike varieties Ude and Galema, varieties Simba and Yerer increased and decreased in similar trend across the treatments (Table 10 and Appendix).

Analysis of variance (Table 10) has shown that there was significant difference among varieties and treatments on induction of embryonic tissues.

Table 10. Effect of 2,4-D and KIN on induction of embryonic tissues using all varieties. Mean values are shown as ± SD.

2,4-D (mg/l)	KIN (mg/l)	Varieties				
		Simba	**Galema**	**Ude**	**Yerer**	**Mean**
0.5	0.0	6.7±2.3	1.7±2.3	2.5±2.5	10.0±0.0	**5.2d**
0.5	1.0	6.7±2.3	2.5±2.5	3.3±1.3	11.6±2.0	**6.0d**
0.5	2.0	9.2±3.4	5.8±1.9	4.2±0.8	11.6±4.7	**7.7cd**
1.0	0.0	20.0±4.0	14.2±1.9	8.3±2.3	16.6±2.4	**14.8ab**
1.0	1.0	26.6±4.6	8.3±2.9	13.3±3.3	35.0±9.4	**20.8a**
1.0	2.0	14.2±5.2	5.8±1.8	9.2±0.8	15.8±3.5	**11.3bc**
1.5	0.0	11.7±4.7	5.0±2.8	8.3±2.4	14.2±4.6	**9.8cd**
1.5	1.0	8.3±3.2	5.0±2.8	8.3±2.4	10.8±1.9	**8.1cd**
1.5	1.0	7.5±2.5	7.5±3.8	6.7±2.4	6.7±2.4	**7.1cd**
2.0	0.0	5.8±1.8	9.2±1.9	7.5±2.7	5.8±2.5	**7.1cd**
2.0	1.0	5.0±1.3	7.5±2.5	5.8±2.5	5.0±2.9	**5.8d**
2.0	2.0	7.5±2.5	5.0±2.8	5.8±2.5	3.3±1.7	**5.4d**
Mean		**10.8ab**	**6.5b**	**6.9b**	**11.2a**	

ANOVA for the effect of 2,4-D and KIN on induction of ETs.

		% ET		
Source of variation	**DF**	**MS**	**F value**	**P**
Variety	3	202.5	3.71	0.0134 *
Treatment	11	278.5	5.11	<0.0001**
Rep	2	25.5	0.47	0.6274
Error	127	54.5		
Total	143	561.0		

Means with the same letter along the row and column are not significantly different at p≥0.05 using Duncan multiple range test. ET= Embryonic tissues*= significant at p≤0.05, **= very significant at p≤0.01.

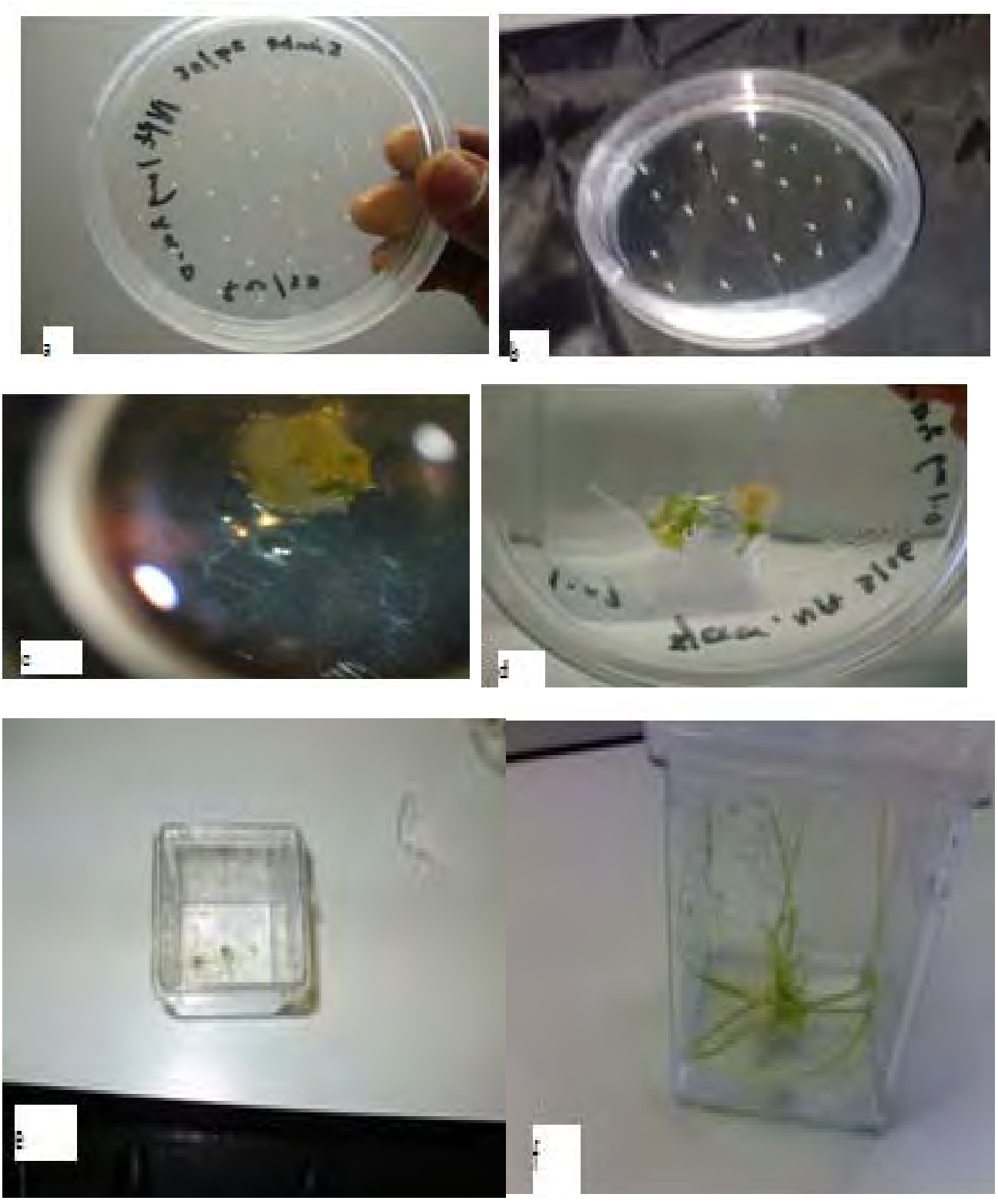

Fig 7. Induced embryonic tissues of varieties Simba and Yerer from their ovary cultures and differentiation of the embryonic tissues into plantlets. a) Ovary culture of variety Simba, b) Ovary culture of variety Yerer, c) Embryonic tissues of variety Simba, d) Embryonic tissues of variety Yerer, e) Plantlets of variety Simba, f) Plantlets of variety Yerer.

Both varieties Ude and Galema induced better percentage of embryonic tissues in the combination of 2 mg/l 2,4-D and 2 mg/l BAP followed by the combination of 1 mg/l 2,4-D and 4 mg/l BAP (Table 11). The average percentage of embryonic tissues were 10.0 % and 8.3 % for varieties Ude and Galema respectively, better than the average percentage of embryonic tissues induced by different concentrations and combinations of 2,4-D and KIN. In 1 mg/l 2,4-D and 8 mg/l BAP, both varieties did not induce embryonic tissues.

Table 11. The effect of different combinations and concentrations of 2,4-D and BAP on induction of embryonic tissues.

Treatments								
Varieties	**2 mg/l 2,4-D + 2 mg/l BAP**		**1 mg/l 2,4-D + 8 mg/l BAP**		**1 mg/l 2,4-D + 4 mg/l BAP**		**1 mg/l 2,4-D + 2 mg/l BAP**	
	Cultured ovaries	% ET	Cultured ovaries	% ET	Cultured ovaries	% ET	Cultured ovaries	% ET
Ude	20	15.0	20	0.0	20	10.0	20	5.0
Galema	20	15.0	20	0.0	20	5.0	20	0.0

ET=Embryonic tissues

5.1.9 The effect of embryo promoting medium on induction of embryonic tissues

Enlarged ovaries were cultured in embryo promoting (EP) medium. After a week, the enlarged ovaries sprouted out embryonic tissues through the micropylar end. The results (Table 12) indicated that varieties of Simba and Yerer did not respond in any of the EP medium. Variety Galema induced better percentage of embryonic tissues (33.3 %) in EP_B medium followed by EP_C medium (16.7 %).

Table 12. The effect of embryo promoting (EP) medium on induction of direct embryogenesis.

Treatments						
Varieties	EP_A = 2.5 mg/l BAP		EP_B = 5 mg/l BAP		EP_C = 10 mg/l BAP	
	Cultured EOs	% ET±SD	Cultured EOs	% ET±SD	Cultured EOs	% ET±SD
Simba	9	0.0±0.0	12	0.0±0.0	9	0.0±0.0
Galema	6	0.0±0.0	6	33.3±0.0	6	16.7±6.4
Yerer	6	0.0±0.0	10	0.0±0.0	6	0.0±0.0

EOs=Enlarged ovaries, ET=Embryonic tissues, EP=Embryo promomoting, SD=Standard deviation

5.1.10 The effect of seasons on induction of embryonic tissues

The effect of seasons on the percentage of induced embryonic tissues from cultured ovaries using all varieties indicated that variety Simba gave the highest percentage (25 %) of embryonic tissues during April-June season followed by variety Yerer (13.8 %) (Fig 8 and Appendix 3). Variety Ude gave the highest percentage of embryonic tissues during July-September season (11.8 %) followed by April-June season (6.3 %) (Appendix 3). Variety Galema induced the maximum percentage of embryonic tissues during January-March season (6.6 %) and minimum during April-June and July-September seasons (1.7 %) (Appendix 3).

Analysis of variance revealed that there was significant difference($P \leq 0.05$) among varieties on induction of embryonic tissues. However, no significant difference ($P \geq 0.05$)was observed among seasons (Table 13).

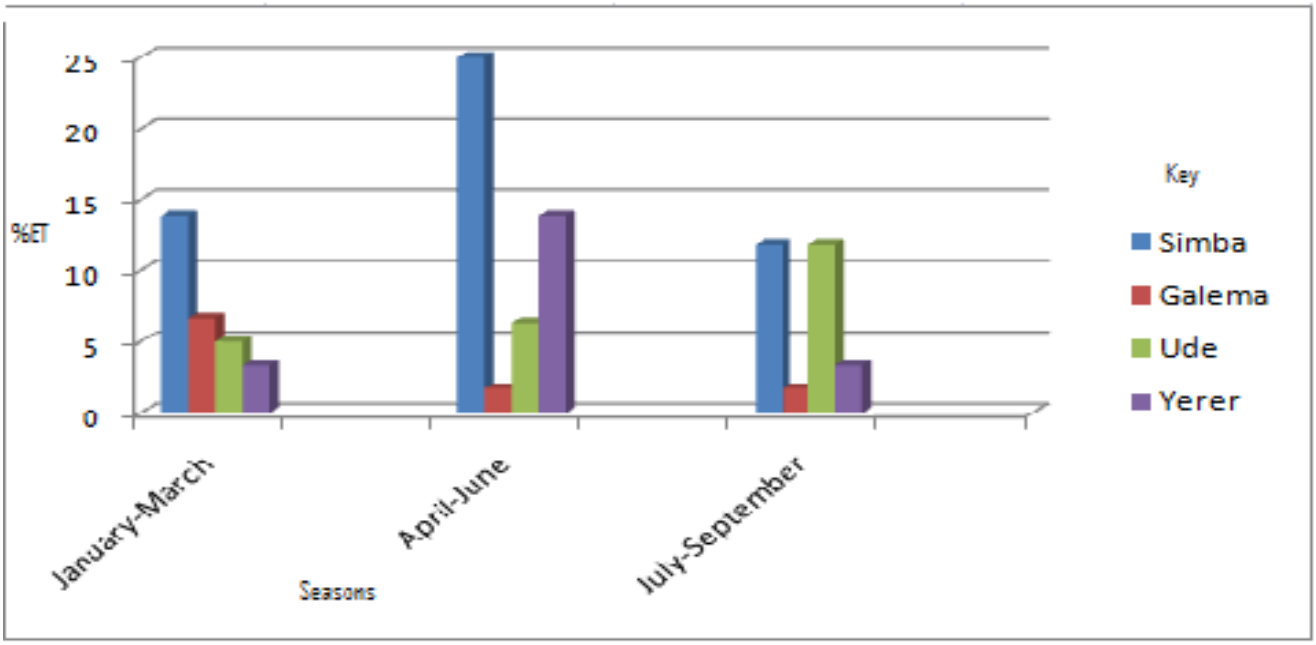

Fig 8. The effect of seasons on the percentage of induced embryonic tissues from ovary cultures of all varieties.

Table 13. Analysis of variance for the effect of seasons on the percentage of induced embryonic tissues.

Source of variation	Induction ET(%)			
	DF	**MS**	**F-value**	**p**
Variety	3	303.1	3.54	0.025*
Seasons	2	121.8	1.42	0.256
Rep	3	143.1	1.67	0.192
Error	32	85.5		
Total	41	653.5		

*=significant difference at p≤0.05.

5.1.11 The effect of PGRs on regeneration of shoots and their growth into full plantlets

A total of 462, 158, 168 and 312 ETs for varieties Simba, Galema, Ude and Yerer were induced on induction media under different experimental conditions, respectively (Table 16). During preliminary observations, 153, 51, 54 and 83 ETs of varieties Simba, Galema, Ude and Yerer respectively were done repeatedly in a different treatments. However, shoots were not regenerated. A total of 29 different treatments that were supplemented to regeneration medium were used. Among these, only 11 treatments were selected and continuously supplemented in the regeneration medium for the regeneration of shoots (Table 14). The following ETs, 309, 107, 114, and 229 of varieties Simba, Galema, Ude and Yerer respectively were cultured in 11 different treatments at different frequencies (Appendix 5 and Table 14).

Maximum responsive embryonic tissues from varieties Simba (41.3 %) and Yerer (41.6 %) were obtained in MS medium supplemented with 0.1 mg/l 2,4-D (Table 14). At this treatment, none of the cultured embryonic tissues regenerated into shoots for varieties Ude and Galema. Galema was the least responsive variety, none of its embryonic tissues regenerated into shoots in all the treatments except in 0.1 mg/l NAA combined with 1 mg/l KIN. This treatment was the only treatment in which the cultured embryonic tissues of the four varieties regenereted into shoots. For varieties Simba 28.0 %, Yerer 25.9 %, Ude 17.6 % and Galema 26.6 % of the embryonic tissues regenerated into shoots. Variety Yerer gave the second and third highest percentage of responsive embryonic tissues in the combinations of 0.05 mg/l 2,4-D with 3 mg/l KIN and 0.1 mg/l NAA with 1 mg/l KIN, respectively. Variety Ude was responsive only in three treatments, 0 mg/l 2,4-D and 0 mg/l KIN (5.8 %), combination of 0.1 mg/l 2,4-D

with 2 mg/l BAP (5.6 %) and combination of 0.1 mg/l NAA with 1 mg/l KIN (17.6 %). Variety Yerer was the most responsive variety for regeneration of shoots followed by variety Simba (Fig 9).

Regeneration of shoots into plantlets was the challenging part of this study. Following the induction of embryonic shoots, they were subcultured in the same regeneration medium once or twice after 2-3 weeks. However, they frequently died (Fig 9 a and b). After many trials, shoots were subcultured on MS medium containing 1 or 1.5 mg/l of GA_3 and 60 g/l sucrose (Fig 11). The medium supplemented with 1.5 mg/l GA_3 gave the longest shoots compared to the medium supplemented with 1 mg/l GA_3 for variety Yerer (Fig 11e). However, very small deviations were observed for variety Simba. Varieties Galema and Ude did not respond in both GA_3 concentrations (Figs 10 and 11). Table 15 indicated that there was significant difference($P \leq 0.01$) among varieties and treatments on the percentage of responsive embryonic tissues. There was no significant difference between treatments, 0.1 mg/l 2,4-D and combination of 0.1 mg/l NAA with 1 mg/l KIN. However, they were significantly different from other treatments on the percentage of shoot regeneration (Table 14). Variety Yerer was significantly different from varieties Ude and Galema, but not significantly different from variety Simba. Variety Simba was not significantly different from other varieties on the percentage of responsive embryonic tissues (Table 14).

As indicated in Table 17, out of a total of 75 regenerants, variety Yerer gave 19 plantlets and 20 shoots having average shoot length of 9.0 cm and root length of 8.5 cm. Variety Simba gave 6 plantlets, 1 root and 19 shoots having average shoot length of 2.0 cm and root length of 6.0 cm. Variety Ude gave 1 pantlet having root length of 4.0 cm and shoot length of 2 cm. It also gave 4 shoots with average length of 2.0 cm. Variety Galema gave only 5 shoots with average length of 3.0 cm. Moreover, Table 16 indicated that variety Yerer gave better percentage of regenerants (1.1 %) from a total of 3,444 cultured ovaries than other varieties. Variety Simba gave the second highest percentage of regenerants (0.55 %) from a total of 4,732 cultured ovaries. Variety Simba gave the maximum percentage of embryonic tissues (9.8 %) followed by variety Yerer (9.0 %). Variety Galema gave the least percentage of induced embryonic tissues and regenerants from a total of 3,360 cultured ovaries (4.7 % and 0.12 %). From a total of 14,524 cultured ovaries using all varieties, 1100 embryonic tissues were induced and 75 regenerants were obtained. The embryonic tissues of 6.8 % and 0.5 % of the cultured ovaries were responsive to the regeneration of shoots or roots or plantlets (Tables 16 and 17). Each embryonic tissue of variety Yerer produced 0-10 plantlets (Fig 9 d, e, g and Fig 11 c). Simba and Ude produced 0-3 (Fig 9 c) and 0-1 plantlets (Fig 10) per embryonic tissue respectively.

Table 14. Effect of PGRs on the percentage of responsive embryonic tissues. Their effect is shown as mean value of % RET±SD.

Treatments		Varieties				Mean of the means
		Simba	Yerer	Ude	Galema	
0.0 2,4-D	0.0 KIN	5.1±1.2	0.0±0.0	5.8±2.4	0.0±0.0	**2.7b**
0.05 mg/l 2,4-D	2.0mg/l KIN	3.3±1.9	23.8±5.5	0.0±0.0	0.0±0.0	**6.8b**
0.05 mg/l 2,4-D	3.0 mg/l KIN	0.0±0.0	29.2±4.1	0.0±0.0	0.0±0.0	**7.3b**
0.1 mg/l 2,4-D	0.0 mg/l KIN	41.3±13.3	41.6±14.4	0.0±0.0	0.0±0.0	**20.7a**
0.1 mg/l 2,4-D	1.0 mg/l KIN	20.6±10.6	0.0±0.0	0.0±0.0	0.0±0.0	**5.2b**
0.1 mg/l 2,4-D	2.0 mg/l KIN	0.04±0.0	0.06±0.0	0.0±0.0	0.0±0.0	**0.0b**
0.1 mg/l 2,4-D	2.0 mg/l BAP	0.042±0.0	9.0±5.0	5.6±2.4	0.0±0.0	**3.7b**
0.1 mg/l NAA	0.0 mg/l KIN	0.0±0.0	4.3±1.1	0.0±0.0	0.0±0.0	**1.1b**
0.1 mg/l NAA	1.0 mg/l KIN	28.0±11.0	25.9±9.8	17.6±6.7	26.6±13.3	**24.5a**
0.1 mg/l NAA	2.0 mg/l KIN	4.2±2.1	0.06±0.0	0.0±0.0	0.0±0.0	**2.1b**
0.5 mg/l IAA	1 mg/l BAP	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**0.0b**
Mean of the means		**9.4ab**	**12.2a**	**2.6b**	**2.4b**	

Table 15. ANOVA for the effect of PGRs on % RET.

Source of variation	% RET			
	DF	MS	F-value	p
Variety	3	1244.4	3.98	0.0087**
Treatment	10	2243.7	7.17	<0.0001**
Rep	12	219.2	0.70	0.7502
Error	224	312.9		
Total	249	4019.5		

Means followed by the same letter showed no significant difference at p≥0.05 using Duncan multiple range test.
****= significant difference, p≤0.01.**

Table 16. The effect of genotypes on the percentage of induced embryonic tissues and regenerants from the total cultured ovaries of each genotype.

Varieties	TCOs	TET	%ET	%RE
Simba	4732	462	9.8	0.55
Galema	3360	158	4.7	0.12
Ude	2988	168	5.6	0.17
Yerer	3444	312	9.0	1.10
Total	**14,524**	**1100**	**7.6**	**0.50**

TCOs= Total cultured ovaries, TET= Total embryonic tissues, % ET= Percentage of embryonic tissues, % RE= Percentage of regenerants.

Table 17. Total regenerants, average root and shoot length of all varieties.

Varieties	Total regenerants	Average root length (cm)	Average shoot length (cm)
Simba	26(1R, 19S, 6Pl)*	6.0	2.0
Yerer	39(20S, 19Pl)*	8.5	9.0
Ude	5(4S, 1Pl)*	4.0	2.0
Galema	5S*	-	3.0
Total	**75**		

***R (root), S (shoot) and Pl (plantlet).**

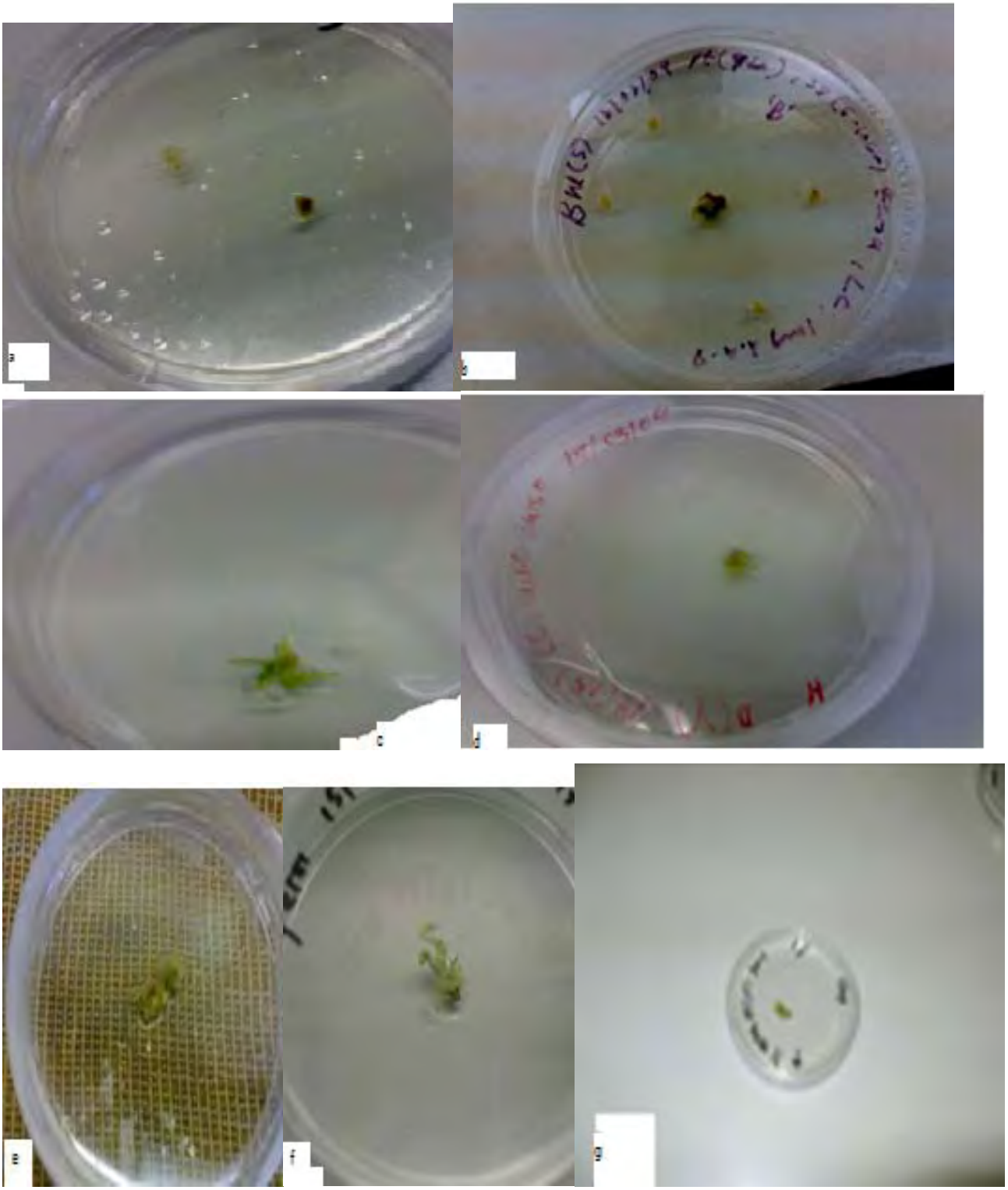

Fig 9. Regenerated shoots from ovary cultures of varieties Simba and Yerer cultured in MS medium supplemented with different concentrations and combinations of PGRs. a) Shoots of variety Simba regenerated in 0.1 mg/l NAA + 2 mg/l KIN combination, b) Shoots of variety Simba regenerated in 0.1 mg/l 2,4-D + 2 mg/l KIN combination, c) Shoots of variety Simba regenerated in 0.1 mg/l 2,4-D + 2 mg/l BAP combination, d) Shoots of variety Yerer regenerated in 0.1 mg/l NAA + 2 mg/l KIN combination, e) Shoots of variety Yerer regenerated in 0.1 mg/l 2,4-D + 2 mg/l KIN combination, f) Shoots of variety Yerer regenerated in 0.1 mg/l 2,4-D, g) Shoots of variety Yerer regenerated in 0.05 mg/l 2,4-D + 3 mg/l KIN combination.

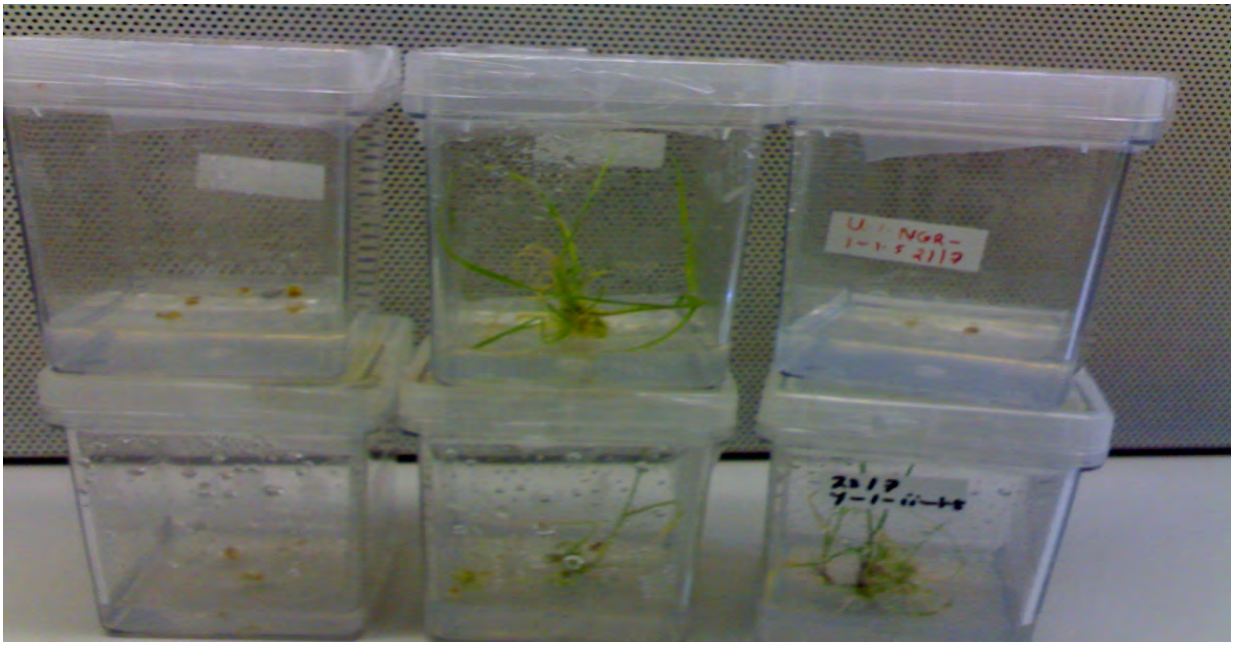

Fig 10. Plantlets of varieties Simba, Ude and Yerer with elongated roots and shoots after 10 weeks starting from the first day of culturing.

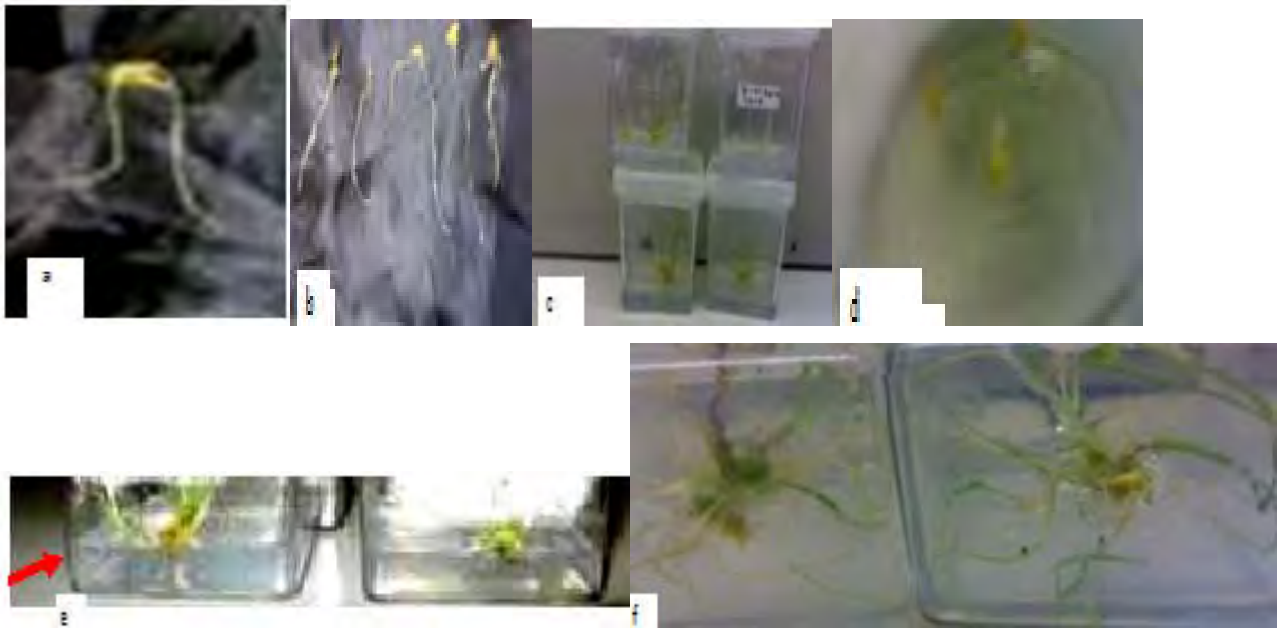

Fig 11. Morphological distinction of the regenerants among varieties cultured in MS medium supplemented with 1.5 mg/l of GA_3. a) Plantlet of variety Ude, b) Plantlets of variety Simba, c) Plantlets of variety Yerer, d) Shoots of variety Galema, e) Plantlets of variey Yerer to verify the effect of 1 and 1.5 mg/l GA_3 on the morphology of regenerants: i) Plantlet of variety Yerer at the left was cultured in 1.5 mg/l of GA_3, ii) Plantlet of variety Yerer at the right was cultured in 1 mg/l GA_3, f) Elongated roots of variety Yerer.

5.1.12 Acclimatization and growth status of plantlets in the growthroom and glasshouse

Two plantlets of variety Simba and 16 plantlets of variety Yerer were successfully transferred into pots containing 3:2:1 proportions of soil, compost and sand (Table 18). Fig 12 indicates, plantlets of variety Yerer just before acclimatization. Each pot was covered with plastic and kept in the growthroom for acclimatization (Figs 13 and 14). However, 4 plantlets of variety Simba, 1 plantlet of variety Ude and 3 plantlets of variety Yerer could not be acclimatized because the plantlets possessed long roots, short shoots and were weak (Fig 11). After 2 weeks, 11 plantlets of variety Yerer were transferred into glasshouse. Only 4 plantlets were survived in the glasshouse for a week. Two weeks later, only one plantlet survived and could not survive until maturity (Fig 15).

Table 18. Number of plantlets transferred into pots, and their subsequent status under growthroom [16 h light (1032-1557 lux) and 8 h dark cycle] and glasshouse under natural light conditions whose temperature was in the range of 38-40 ^{o}C.

		Status of plantlets in growthroom and glasshouse	
Varieties	**No. of plantlets Transferred into pots**	**No. of plantlets survived in the growthroom**	**No. of plantlets Survived in the glasshouse**
Yerer	16	11	1
Simba	2	0	0
Total	**18**	**11**	**1**

Fig 12. Plantlets of variety Yerer just before acclimatization.

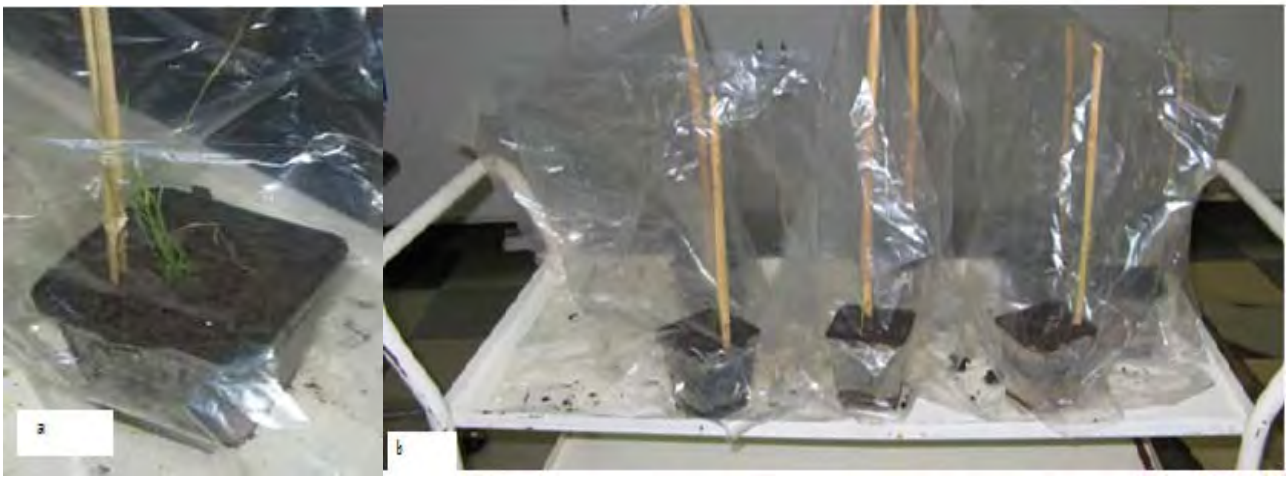

Fig 13. Acclimatization of plantlets in the growthroom. a) Acclimatization of single plantlet containing tillers, b) First round acclimatization of varieties Yerer and Simba together in the growthroom.

Fig 14. Second round acclimatization of plantlets of variety Yerer in the growthroom. a) Acclimatized plantlets of variety Yerer covered with plastic for 2 weeks, b) Survived plantlets of variety Yerer after 2 weeks in the growthroom.

Fig 15. Growth of plantlets of variety Yerer after acclimatization. a) Plantlet of variety Yerer after 10 days of acclimatization, b) Plantlets of variety Yerer grown in the glasshouse, c) Plantlet of variety Yerer after a month in a glasshouse.

5.2 EMBRYO RESCUE CULTURES OF TEF

5.2.1 Determination of the developmental stage of immature embryos of F_1 hybrids on induction of somatic embryos

The effect of different developmental stages of immature embryos on the percentage of induced somatic embryos indicated that florets cultured 6 days after artificial crossing produced the maximum percentage of somatic embryo (60 %) followed by 3 and 9 days after artificial crossing that produced the same frequency (20 %) of somatic embryos. Therefore, 6 days after artificial crossing was taken as the optimum developmental stage of immature embryos for induction of somatic embryos and used for other experiments (Table 19).

Table 19. The effect of developmental stage of immature embryos on induction of somatic embryo (%) using F_1 hybrid of *E. pilosa* (30-5)*Kaye Murri.

3 daac			**6 daac**			**9 daac**			**12 daac**		
CF	SE	% SE	CF	SE	% SE	CF	SE	% SE	CF	SE	% SE
5	1	20.0	5	3	60.0	5	1	20.0	5	0	0.0

daac=days after artificial crossing, CF=cultured florets, SE= somatic embryo

5.2.2 The effect of 2,4-D on induction of somatic embryogenesis

The effect of different concentrations of 2,4-D on induction of somatic embryos was presented in Table 20. The results indicated that the F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri induced maximum percentage of somatic embryos in 1 mg/l 2,4-D (46.7 %). The F_1 hybrids of *E. pilosa* (30-5)*DZ-Cr-37 induced somatic embryos only in 0.1 mg/l 2,4-D (13.3 %). However, the F_1 hybrids of *E. pilosa* (30-5)*DZ-01-196, *E. pilosa* (30-5)*DZ-Cr-387, *E. pilosa* (37 80 87)*Kaye Murri, *E. pilosa* (37 80 87)*DZ-Cr-387, *E. pilosa* (37 80 87)*DZ-Cr-37, *E. curvula**DZ-Cr-387 did not induce somatic embryos in any of the 2,4-D concentrations.

As a whole, there was significant difference among treatments and parental combinations indepedently on induction of somatic embryos (Table 21). The parental combination of *E. Pilosa* (30-5)*Kaye Murri was significantly different from the other seven parental combinations. The remaining seven parental combinations were not significantly different among themselves on induction of somatic embryos. There was no significant difference among the treatment of 1 mg/l 2,4-D and treatments of 0.5 mg/l 2,4-D and 0.1 mg/l 2,4-D. However, it was significantly different from other treatments. Treatments of 0.1 mg/l 2,4-D and 0.5 mg/l 2,4-D were not significantly different from 1 mg/l of 2,4-D and other treatments on induction of somatic embryos (Table 20).

Table 20. Effect of 2,4-D on induction of somatic embryos. Mean values are shown as ± SD.

Parental combination	Treatments						
	0 mg/l 2,4-D	0.1 mg/l 2,4D	0.5 mg/l 2,4-D	1 mg/l 2,4-D	1.5 mg/l 2,4-D	2 mg/l 2,4-D	Mean
E. pilosa (30-5)* Kaye Murri	0.0±0.0	13..3±5.5	20.0±0.0	46.7±9.4	13..3±5.5	0.0±0.0	**15.6a**
E. pilosa (30-5)*DZ-Cr-37	0.0±0.0	13.3±5.5	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**2.2b**
E. pilosa (30-5)*DZ-01-196	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**0.0b**
E. pilosa (30-5)*DZ-Cr-387	0.0±0.0	-	-	0.0±0.0	0.0±0.0	-	**0.0b**
E. pilosa (37 80 87)*Kaye Murri	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**0.0b**
E. pilosa (37 80 87)* DZ-Cr-387	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**0.0b**
E. pilosa (37 80 87)* DZ-Cr-387	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**0.0b**
*E. curvula**DZ-Cr 387	-	-	0.0±0.0	0.0±0.0	-	-	**0.0b**
Mean	**0.0b**	**3.3ab**	**2.5ab**	**5.8a**	**1.7b**	**0.0b**	

Table 21. ANOVA for the effect of 2,4-D on induction of somatic embryo.

Source of variation	DF	MS	F-value	P
Parental combination	7	533..3	12.83	<0.0001**
Treatment	5	117.8	2.83	0.0184*
Rep	2	2.8	0.07	0.94
Error	129	41.6		
Total	143	695.5		

Means followed by the same letters in the same row or column at p≤0.05 are not significantly different.
***=significant difference at p≤0.05, **=significant difference at p≤0.01.**

5.2.3 Regeneration of plantlets from somatic embryos of F_1 hybrids of tef with its wild relative

After 5-6 weeks, from a total of 635 cultured florets of F_1 hybrids, 21 somatic embryos (3.3 %) of F_1 hybrids were obtained. Of which 19 somatic embryos were from the cross of *E. pilosa* (30-5) with Kaye Murri and 2 somatic embryos were from the cross of *E. pilosa* (30-5) with DZ-Cr-37. Somatic embryos (Fig 16c) were transferred into regeneration medium, half strength MS medium without PGRs supplemented with 30 g/l of maltose. Embryos were differentaited into 1-5 shoots per explant (Figs 16d, e and 17). During the third subculturing, shoots were subcultured in the same medium and induced numerous roots (Fig 16g). Out of 19 somatic embryos of *E. pilosa* (30-5)*Kaye Murri, 14 somatic embryos (73.7 %) were differentaited into shoots. A single somatic embryo could give 1-10 plantlets (Fig 16h). From 2 somatic embryos of *E. pilosa* (30-5)*DZ-Cr-37, only 1 shoot was regenerated (Fig 17).

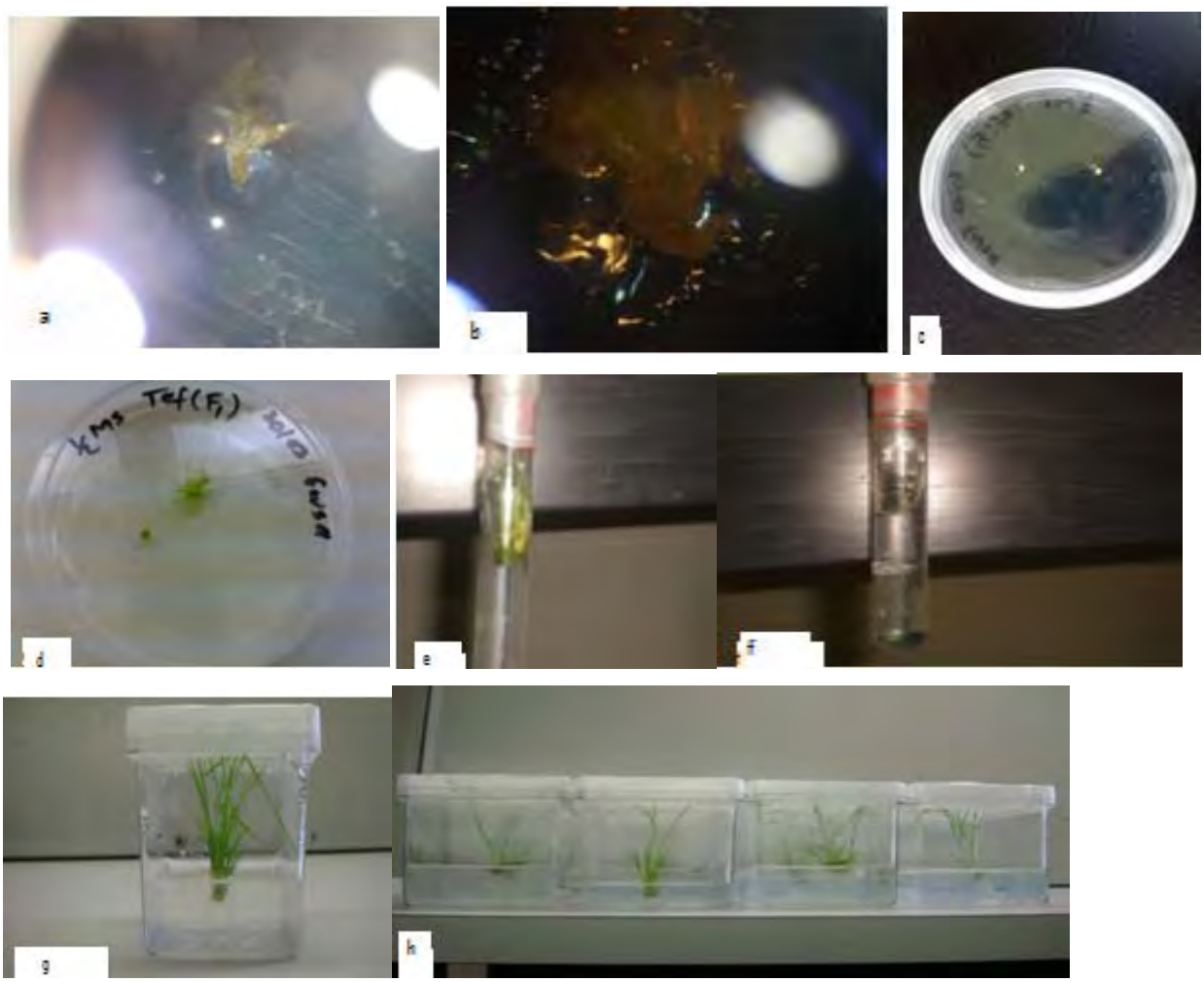

Fig 16. Sequencial growth of F_1 hybrids of tef *(Eragrostis tef)* with *Eragrostis pilosa* (30-5). a) Floret culture of F_1 hybrid excised from the panicle, b) Pollinated ovary of the hybrid ready for excision from the floret after three weeks, c) Somatic embryo of the hybrid excised from pollinated ovary culture after six weeks, d) Shoots regenerated from somatic embryo of the hybrid in regeneration medium (half MS without PGRs) after nine weeks, e) Shoots were subcultured in the same medium and bunch of shoots were regenerated after 11 weeks, f) A single shoot regenerated from a somatic embryo in the same condition as e, g) A bunch of shoots and roots from a single somatic embryo, after the third subculturing on the same regeneration medium, h) Plantlets of the hybrid showing variation in the number of shoots and roots.

Fig 17. a) Shoot of F_1 hybrid of *E. pilosa* (30-5)*DZ-Cr-37 after five weeks in a regeneration medium (half strength MS), b) Shoot of F_1 hybrid of *E. pilosa* (30-5)*DZ-Cr-37 after 8 weeks of subculturing in the same medium.

5.2.4 Acclimatization of plantlets and growth status in the growthroom and glasshouse

Twenty four plantlets of F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri were successfully transferred into pots, covered with plastic and kept in the growthroom for ten days (Fig 18) for acclimatization as described in unpollinated ovary cultures of wheat. All plantlets were survived in the growthroom and transferred into glasshouse. In the glasshouse, after three days, only three plantlets were died due to fungal contamination in the growthroom. As indicated in Fig 19a, twenty one plantlets were survived in the glasshouse. They were grown to maturity in the same glasshouse (Figs 19b and 22). The F_1 hybrids share qualitative and quantitative characteristics from both parents (Fig 20). For instance, panicle color, seed color, vasal stock color and other characteristics of the hybrids may be derived from Kaye Murri. Panicle form, peduncle length, height and other characteristics may be derived from *E. pilosa* (30-5). At maturity, each plantlet in a pot induced 8-40 tillers (Fig 23), 21 pots together induced a total of 442 single plantlets (Table 22 and Fig 23). All plantlets of the F_1 hybrid were green and fertile (Fig. 21). Some quantitative characteristics of the main tillers were evaluated (Table 22). According to the results, the average plant height, panicle length, peduncle length and culm length were 93.3±12.1, 30.2±3.4, 21.9±5.5 and 63.2±9.8, respectively.

Table 22. Morphological characteristics of rescued hybrid of *E. pilosa* (30-5) vs *E. tef* cv. Kaye Murri.

Characteristics of the hybrids					
Plant in pot no.	Plant height (cm)	Panicle length (cm)	Peduncle length (cm)	Culm length (cm)	No. of tillers/plant
1	93	32	17	61	13
2	110	34	32.6	76	9
3	86	31	19.5	55	21
4	82	29	21.3	53	21
5	117	35	33.5	82	8
6	101	32	24.5	69	10
7	85	31	25	54	14
8	107	36	13.5	71	16
9	104	31	21	73	16
10	87	30	24.6	57	33
11	92	22	29.7	70	10
12	108	29.7	28	78.3	15
13	65	22.5	17	42.5	27
14	90	28.5	17	61.5	27
15	87	26	17	61	24
16	93	31	15	62	38
17	100	34.5	23	65.5	30
18	99	34	22.5	65	32
19	77	27	15.1	50	20
20	96	29	22	67	40
21	81	28.5	22	52.5	18
Average	93.3±12.1	30.2±3.4	21.9±5.5	63.2±9.8	T.no.of single plants=442

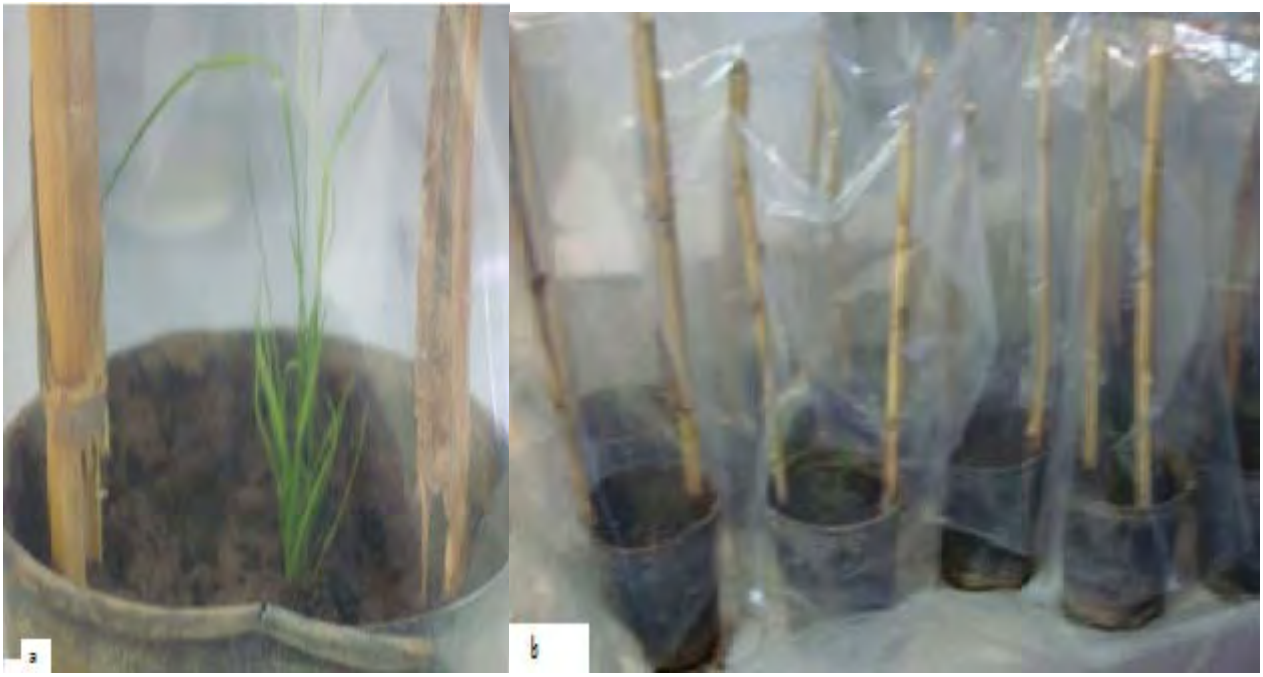

Fig 18. Plantlets of F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri acclimatized after 15 weeks. a) Single plantlet acclimatized in growthroom, b) 12 plantlets were acclimatized for the first time.

Fig 19. Growth of plantlets of the F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri in the glasshouse. a) After two weeks transferred in the glasshouse, b) At the heading stage.

Fig 20. Regenerated F_1 hybrid of *E. pilosa* (30-5)*Kaye Murri with its parents: i) Left, cv. Kaye Murri , ii) middle, F_1 hybrid iii) Right, *E. pilosa* (30-5).

Fig 21. All the plantlets of the F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri grown in the glasshouse were green and fertile.

Fig 22. Plantlets of the F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri that were grown to maturity in the glasshouse. Their height is with the range of 65-117 cm.

Fig 23. A single plantlet of F_1 hybrid of *E. pilosa* (30-5)*Kaye Murri, induced 40 tillers.

6. DISCUSSION

6.1 UNPOLLINATED OVARY CULTURES

6.1.1 The effect of types of explants on gynogenic response

Explant types are among the factors that affect gynogenic response (Campion *et al.*, 1992; Likyelesh Gugsa *et al*., 2006). Except variety Simba, ovary cultures of varieties Galema, Ude and Yerer gave better percentage of developed ovaries than spike and spikelet cultures. For variety Simba, ovary cultures induced better percentage of embryonic tissues (1.3 %) than spike (0.0 %) and spikelet cultures (0.0 %). The percentage of embryonic tissues of varieties Galema, Ude and Yerer were similar for the three types of explants (0.0 %). However, in onion (*Allium cepa* L.), similar results have been reported (Campion *et al.,* 1992). Ovary cultures gave better number of embryos than flower cultures (Campion *et al.,* 1992). In contrast, in mulbery (*Morus alba* L.), the percentage of ovaries showing preembryo induction during inflorescence segment cultures was much higher than that of ovaries producing gynogenic plants during isolated ovary cultures (Thomas, 2004). Likyelesh Gugsa *et al*. (2006) have also reported that unpollinated pistil cultures of tef (*Eragrostis tef*) gave poor gynogenic response than spikelet and panicle cultures.

6.1.2 The effect of light and dark on the development of ovaries and induction of embryonic tissues

Gynogenic plants may be regenerated through direct embryogensis and/or indirectly via callusing phase. Regeneration of gynogenic plants through direct embryogenesis was reported in unpollinated ovary and flower cultures of onion (Campion *et al.,* 1992); unpollinated ovary cultures of durum wheat (Sibi *et al*., 2004); unpollinated ovary and inflorescence segment cultures of mulbery (Thomas, 2004); unpollinated pistil, panicle and spikelet cultures of tef (Likyelesh Gugsa, 2005; Likyelesh Gugsa *et al*., 2006) and unpollinated ovule cultures of niger (Bhat and Murthy, 2007). In the present study, similar phenomenon was observed. Embryos were sprouting out from unpollinated ovaries following their development within 1-2 weeks. On the other hand, in durum wheat Alaoui *et al*. (1998) and Slama-Ayed and Slim-Amara (2007) reported regeneration of gynogenic plants from unpollinated ovary cultures through callusing phase. Likyelesh Gugsa (2005) has reported that the presence or absence of light has no effect on the enlargement of pistils. However, in the presnt work it has been observed that the effect of light has much influence on formation of embryonic tissues in all genotypes cultured under this condition. In the present study, light and dark culture conditions were used. Ovaries were enlarged in both dark and light culture conditions. Only variety Galema induced embryonic tissues (1.6 %) under dark culture condition. Other three varieties did not induce embryonic tissues under this culture condition. As compared to dark

culture condition, under light culture condition, all the four varieties induced better percentage of embryonic tissues. Thus, the results described in this study were in harmony with the work of Likyelesh Gugsa (2005).

6.1.3 Determination of appropriate harvesting stage of spike

The stage at which the explants were cultured is critical step for induction of embryonic tissues (Thomas, 2004; Likyelesh Gugsa, 2005). After preliminary observations, appropriate harvesting stage of spikes that could give the best gynogenic embryos was determined by taking three stages using variety Simba. Ovaries cultured from stage II gave the best percentage of embryonic tissues (25 %) followed by stage I (11.6 %). However, ovaries cultured from stage III gave least percentage of embryonic tissues (5 %). There was significant difference among the stages on induction of embryonic tissues. The study in rice by Zhou *et al*. (1986) have revealed that gynogenesis did not trigger until the cultured immature ovaries reached gametophyte stage. This might correspond to stage I of the present study. In barely, large number of callus and haploid plants were induced from unpollinated ovary cultures at trinucleate stage (Castillo and Cistu, 1993). Niger (Bhat and Murthy, 2007) and squash (Shalaby, 2007) mature ovules collected one day before anthesis or on the day of anthesis induced better number of gynogenic embryos. This might be in line with stage III of the present study. Pistilate cultures of tef varieties did not show gynogenic response when the ovaries were matured and immatured (Likyelesh Gugsa, 2005). Sibi *et al*. (2004) have reported that better gynogenic response was found when the spikes were harvested at bi- or trinucleate stage of microspores of wheat. Slama-Ayed and Slim-Amara (2007) reported the highest regeneration frequency of gynogenic response from unpollinated ovary cultures of durum wheat. This was obtained when the microspore population was in late mononucleate to binucleate stages. These reports may be in consistent with stage II of the present study.

6.1.4 The effect of cold pretreatment durations on induction of embryonic tissues

Induction of embryonic tissues in unpollinated ovary cultures of wheat were dependent on cold pretreatment. Seven days of cold pretreatment were taken as the optimum for induction of embryonic tissues (Sibi *et al*., 2004). A cold stress treatment of microspore cultures of maize at 8 to 9 °C for 10 to 14 d was optimum for androgenic culture (Aulinger, 2002). A thermal pretreatment of 5-15 d at 5 °C was also favorable for gynogenesis of durum wheat (Alaoui *et al*., 1998). Slama-Ayed and Slim-Amara (2007) have also reported that in durum wheat, 14 days pretreatment at 4 ^{o}C gave the highest response of ovary development and callus induction. All the above reports were in agreement with the present study. For all varieties, better number of gynogenic embryos were induced at 15 d of cold pretreatment (4 ^{o}C) followed by 10 d. There was significant difference among varieties and pretreatment durations on

induction of embryonic tissues independently. On the other hand, in squash, ovules incubated at 4 or 32 °C for 4 days produced better embryonic response (Shalaby, 2007). In tef, Likyelesh Gugsa (2005) reported that although gynogenic response was not highly affected by cold pretreatment (4 ^{o}C), the efficiency increased with cold pretreatment for short duration. For instance, cold pretreatment at 4 ^{o}C for 36 h improved the formation of gynogenic tissues in panicle and spikelet cultures of tef.

6.1.5 The effect of sucrose and maltose on induction of embryonic tissues

Sucrose or maltose were used for an induction medium at three levels (30, 60 and 90 g/l). Maltose at 30 g/l was found to be the optimum sugar type at a given concentration. This concentration of maltose was taken as the best sugar source for callus induction of F_1 hybrids of rice (Tsuchiya, 1992). Mulu Ayele and Helmut (1995) conducted a similar experiment as the present study in anther and inflorescence cultures of tef. They found that 30 g/l maltose was the optimum. Their experiment was in line with the present study. On the contrary, 30 g/l sucrose was used as optimum sugar source for the induction media of ovule cultures of niger (Bhat and Murthy, 2007) and squash (Shalaby, 2007).

6.1.6 The effect of culture media on induction of embryonic tissues

MS (Murashige and Skoog, 1962) medium supplemented with different growth regulator combinations and concentrations has been commonly used as induction and regeneration medium in different crops. Immature embryo culture of durum wheat (Sears and Deckard, 1982), anther cultures of rice (Cho and Zapata, 1990), mature seed culture of tef (Kebebew Assefa *et al*., 1998), gynogenic culture of mulbery (Thomas, 2004), immature embryo cultures of durum wheat (Satyavathi *et al*., 2004), spike and immature embryo cultures of bread wheat (Lin Shu *et al*., 2006) were some of them. It was also reported in pistil, panicle and spikelet cultures of tef (Likyelesh Gugsa, 2005), squash gynogenic ovules (Shalaby, 2007) and unpollinated ovule cultures of niger (Bhat and Murthy, 2007). N_6 medium was also used for gynogenic response of barely unpollinated ovary cultures (Castillo and Cistue, 1992) and in anther cultures of rice (Tsuchiya, 1992). Moreover, B_5 (Gamborg *et al*., 1968) medium was the best medium for plant regeneration in comparison with MS, NN (Nitsch and Nitsch, 1969) and N_6 (Chu, 1978) media from cultured ovules of niger (Bhat and Murthy, 2007). In the present study, three types of induction media (MS, N_6, and B_5) were compared. MS was found to be the best induction medium and the only medium used for regeneration. The results were in agreement with the above reports.

6.1.7 The effect of PGRs on induction of embryonic tissues

In unpollinated ovary cultures of durum wheat, 2,4-D was necessary for callus formation (Alaoui *et al*., 1998). The combination of Kinetin and 2,4-D was evaluated for callus induction of immature embryo

cultures of wheat (Jones *et al*., 2005**)**. In ovule cultures of *Lolium multiflorum* (Kumlehn and Nitzsche, 1995) and squash (Shalaby, 2007), 1 mg/l from each of kinetin and 2,4-D was the optimum PGR combination for induction of embryonic tissues. In immature embryo cultures of bread wheat, callus was initiated by 1 mg/l 2,4-D. Calli were initiated after 2 weeks and variability was observed among the wheat genotypes tested for callus induction (Sears and Deckard, 1982). Spike and immature embryo cultures of bread wheat at 2 mg/l 2,4-D was the optimum growth regulator for callus induction (Lin Shu *et al*., 2006). In seedlings, roots and stem pieces of common wheat, callus growth was also vigorous when 0.5-2.0 mg/1 2,4-D was added (Shimada *et al*., 1969). In the present study, 12 concentrations of 2,4-D alone (0.5-2 mg/l) or in combination with 1 or 2 mg/l KIN were supplemented in MS medium as induction medium and 1 mg/l of each of 2,4-D and KIN was the most effective PGR combination for all genotypes except for variety Galema. The second highest PGR concentration for the induction of embryonic tissues except for varieties Galema and Ude was 1 mg/l of 2,4-D. There was significant difference among treatments and genotypes for induction of embryonic tissues which is in agreement with most of the above reports.

Since ovary cultures of varieties of Ude and Galema induced small percentage of embryonic tissues in 2,4-D and KIN combinations, other four 2,4-D and BAP combinations were used to induce better percentage of embryonic tissues. The optimum embryonic tissues were induced in 2 mg/l of each BAP and 2,4-D. This particular investigation was in agreement with the work of Likyelesh Gugsa *et al*. (2006) for panicle and spikelet cultures of tef. However, they could not regenerate into shoots.

6.1.8 The effect of embryo promoting medium on induction of embryonic tissues

In the present study, although most of the cultured ovaries enlarged after 2-3 weeks for all varieties, not all of them developed embryonic tissues. To increase the chance, embryo promoting medium was used for varieties Simba, Galema and Yerer. BAP at the concentrations of 2.5, 5 and 10 mg/l were used to promote embryonic tissues from enlarged ovaries. Kebebew Assefa *et al*. (1998) used EP medium to induce somatic embryos from calli of tef derived from mature seed cultures. The authors found somatic embryos in all of the PGR combinations. However, the PGR combination in the present study was different from the work of Kebebew Assefa *et al*. (1998). Only the principle was applied and successful result was obtained from variety Galema. Its embryonic tissues induced in EP_B and EP_C. However, enlarged ovaries of varieties Simba and Yerer did not induce embryonic tissues in any of the embryo promoting medium.

6.1.9 The effect of seasons on induction of embryonic tissues

Doctrinal *et al*. (1989) further demonstrated that June was the most favorable of all the summer months for gynogenesis in cultivars of *Beta vulgaris*. Both physiological state of the donor plants and growing seasons influence gynogenesis in tef (Likyelesh Gugsa, 2005). Panicles taken in winter season (January-March) were more responsive than summer (Likyelesh Gugsa, 2005). In durum wheat, the comparison of the two experiments, spring and summer seasons, showed that summer was the best season for *in vitro* unpollinated ovary culture (Sibi *et al*., 2004). According to the present investigation, April-June was more responsive season for the induction of embryonic tissues for varieties Simba and Yerer. July-September was more responsive season for variety Ude. There was significant difference among varieties and no significant difference among seasons on the induction of embryonic tissues. Therefore, the present study was in agreement with the work of Doctrinal *et al*. (1989) cited in Thomas (2004), partly in agreement and partly not in agreement with the works of Sibi *et al*. (2004) and not in agreement with the work of Likyelesh Gugsa (2005). This difference might be due to tef and wheat belong to different subfamilies. Genotypes of wheat used for the work of Sibi *et al*. (2004) and the present study were different.

6.1.10 The effect of genotypes on the induction of embryonic tissues and regeneration of plantlets

Genotypes are key factor influencing induction of embryonic tissues in ovary cultures of squash (Shalaby, 2007) and in unpollinated ovary cultures of durum wheat (Sibi *et al*., 2004). In the present study also, the induction of embryonic tissues were dependent on genotypes.

Immature embryo cultures of wheat (Sears and Deckard, 1982), gynogenic cultures of onion (Campion *et al*., 1992), ovule cultures of *Lolium multiflorum* (Kumlehn and Nitzsche, 1995), ovary and inflorescence segment cultures of mulbery (Thomas, 2004), unpollinated ovary cultures of durum wheat (Sibi *et al*., 2004), immature embryo cultures of durum wheat (Satyavathi *et al*., 2004), spike and immature embryo cultures of bread wheat (Lin Shu *et al*., 2006), gynogenic cultures of squash (Shalaby, 2007**)** and ovary cultures of durum wheat (Slama-Ayed and Slim-Amara, 2007) revealed that plantlet regeneration was influenced by genotypes. In the present study, regeneration of plants from ovary cultures of wheat depended up on genotypes which were in comformity with the above reports.

Thirty-six percent of the embryos obtained from gynogenic cultures of onion could regenerate whole plantlets and 88.3 % of the regenerants were haploid (Campion *et al*., 1992). Plantlet regeneration was observed in ovule cultures of *Lolium multiflorum* in the range of 43.5-57.8 % (Kumlehn and Nitzsche, 1995). Eighty-four plants (0.7 %) were obtained from a total of 12,000 cultured unpollinated ovaries of

wheat (Slama-Ayed and Slim-Amara, 2007). In mulberry, a maximum plant regeneration of 16 % was obtained (Thomas, 2004). Squash showed the highest percentage of responding ovules with 48.8 % and an ovule producing three plantlets (Shalaby, 2007). In anther cultures of durum wheat, from a total of 86,400 cultured anthers, 324 (0.38 %) plants were obtained. Out of 324 plantlets, 248 were green plants (0.29 %) and 76 were albino plants (0.09 %). Only 3 of the 10 genotypes (42.58 %) produced green plants (Doramaci-Altuntepe *et al*., 2001). In microspore cultures of bread wheat, 83 (27.2 %) were green and 222 (72.8 %) were albino plants (Kim *et al*., 2003). In the present study, ovary cultures of three genotypes from the four wheat genotypes (75 %) induced green plants which were better than the anther cultures of durum wheat (Doramaci-Altuntepe *et al*., 2001). They induced a minimum and a maximum regenerants of 0.12 % and 1.1 % from ovary cultures of varieties Galema and Yerer respectively. From a total of 14,524 cultured ovaries using four varieties, 75 regenerants (0.50 %) were obtained which were better than the work of Doramaci-Altuntepe *et al*. (2001) and closer to the work of Slama-Ayed and Slim-Amara (2007). All the plantlets were green and no albino plants were obtained. This is considered as an advantage over androgenic cultures reported by Doramaci-Altuntepe *et al*. (2001) and Kim *et al*. (2003). Depending on genotypes each cultured ovary could regenerate from 0-10 plantlets which was much better than the reports of Shalaby (2007) on ovule cultures of squash.

6.1.11 The effect of plant growth regulators on regeneration of shoots and their growth into full plantlets

In unpollinated ovary cultures of durum wheat, 2,4-D was necessary for green plant regeneration (Alaoui *et al*., 1998). In mature embryo cultures of bread wheat, the calli of all the cultivars were transferred into regeneration medium supplemented with 0.5 mg/l IAA and 1 mg/l BAP (Fazelienasab *et al.*, 2004). In *Lolium multiflorum* ovules, highest frequencies of plantlet regeneration per number of cultivated ovule were recorded at 0.2 mg/l 2,4-D combined with 0.2 mg/l benzyl adenine containing medium (Kumlehn and Nitzsche, 1995). In immature embryo cultures of wheat, shoots were initiated by reducing the 2,4-D from 1 mg/l to 0.1 mg/l. Complete plants were regenerated by transferring into 2,4-D free medium (Sears and Deckard, 1982). In the present study, 11 combinations and concentrations of PGRs were taken and significant difference was obtained among treatments on shoot regeneration. The first, second and third highest PGR combinations for shoot regeneration of variety Simba were 0.1 mg/l 2,4-D, 0.1 mg/l 2,4-D + 1 mg/l KIN and 0.1 mg/l NAA + 2 mg/l KIN respectively. The first to fourth best PGR combinations for shoot regeneration of variety Yerer were 0.1 mg/l 2,4-D, 0.05 mg/l 2,4-D + 3 mg/l KIN, 0.1 mg/l NAA + 1 mg/l KIN and 0.05 mg/l 2,4-D + 2 mg/l KIN. The best PGR combination for shoot induction of varieties Ude and Galema was 0.1 mg/l NAA + 2 mg/l KIN. The regenerated shoots were subcultured in auxin free MS medium supplemented with 0-1.5 mg/l of GA_3. To some extent, these results were in

agreement with the works of Sears and Deckard (1982). Ozias-Akins and Vasil (1982) reported that 1-3 mg/l KIN was supplemented for regeneration media. However, it could not be used as shoot regeneration media alone. In contrast to the works of Mulu Ayele and Helmut (1995) and Rashid *et al*. (2009), shoots were not regenerated in BAP and IAA containing medium.

Thirty gram per liter of sucrose in rice anther derived calli (Tsuchiya, 1992) and ovule cultures of niger (Bhat and Murthy, 2007), 60 g/l sucrose in embryo cultures of Saffron (Shiebani *et al*., 2007), 80 g/l sucrose in spike and immature embryo cultures of bread wheat (Lin Shu *et al*., 2006) and 90 g/l sucrose in ovary cultures of barely (Castillo and Cistue, 1992) were used as optimum carbon source for plant regeneration. In the present study, instead of 30 g/l maltose or sucrose, 60 g/l sucrose was used for regeneration of shoots and full plantlet development. This study was similar to the work of Shiebani *et al*. (2007) and in contrast to the works of Tsuchiya (1992) and Bhat and Murthy (2007).

In embryo cultures of switchgrass, embryogenic calli were transferred into MS medium supplemented with 0.5 mg/l GA_3 for plant regeneration (Somleva *et al*., 2002). Microspore cultures of durum wheat presently preferred concentration range for gibberellin most preferably from about 0.2 mg/l to about 4.0 mg/l (Konzak *et al*., 1999). Addition of 1 mg/l GA_3 in the germination medium improved the germination of embryos by 1.5 % compared to the control. Moreover, GA_3 breaks dormancy in seed germination (Likyelesh Gugsa, 2005). In the present study, 1.5 mg/l GA_3 was best PGR for elongation of shoots which was in the range proposed by (Konzak *et al*., 1999) and exceeds by 0.5 mg/l from the work of Likyelesh Gugsa (2005).

6.1.12 Growth conditions of regenerants

The handling of regenerants should done because the *in vitro* environment is different from ambient environmental conditions. Out of 18 green plantlets that were transferred into pots, 68.8 % of the regenerants survived and 32.2 % of the regenerants died in the growthroom. Out of 11 plantlets of variety Yerer, four plantlets (36.4 %) survived for a week in the glasshouse. Only one plantlet survived for two months. However, its growth did not show morphological distinctions throughout its life span. The death of plantlets in the growthroom might be due to inappropriate soil type and composition, high temperature when pawer was off (29-30 ^{o}C) and fungal contaminations. The death of plantlets in the glasshouse was mainly due to high temperature of the glasshouse (38-40 ^{o}C). As reported by Leone *et al*. (2006) the temperature of the glasshouse for wheat should have been between 21-25 ^{o}C. Thus, the temperature of the growthroom as well as glasshouse should be adjusted in order to increase the chance of survival of plantlets both in the growthroom and glasshouse.

6.2 EMBRYO RESCUE CULTURES

6.2.1 Determination of developmental stage of immature embryos of F_1 hybrids

Developmental stage is one of the prerequisite factor for the induction of somatic embryos (Kebebew Assefa *et al*., 1998; Likyelesh Gugsa, 2005; Korbin *et al*., 2008). Pollinated florets from four developmental stages (3, 6, 9 and 12 days) after artificial crossing (daac), were taken to identify the most responsive developmental stage of immature embryos for induction of somatic embryos. The study has found that 6 daac was the best developmental stage of immature embryos for induction of somatic embryos which was in agreement with immature pollinated pistilate cultures of tef (Likyelesh Gugsa, 2005). On the other hand, in ovule cultures of lily, 10 days after interspecific crosses, embryo could be germinated (Chi, 2002). In immature zygotic embryo cultures of tef 10-12 days after pollination were the optimum (Likyelesh Gugsa, 2005). In zygotic embryo cultures of bread wheat, 12-16 days post-anthesis were the optimum (Jonsson, 1995; Jones *et al*., 2005**)**. In pollinated ovule cultures of barely, ovules contained a poorly developed embryo after 10-12 days (Töpfer and Steinbiss, 1985). The types of explant taken during the first culture might be accounted for the differences.

6.2.2 The effect of 2,4-D on induction of somatic embryo

Somatic embryos can be obtained either via direct or indirect somatic embryogenesis (Likyelesh Gugsa, 2005; Hussien *et al*., 2006). In direct embryogenesis, the embryos originate directly from tissues in the absence of conspicuous callus proliferation (Hussien *et al*., 2006). Induction of somatic embryos directly from plant tissue is the most desirable approach in order to avoid somaclonal variation (Aoshima, 2005 cited in Hussien *et al*., 2006). Furthermore, it reduces the time required for plant regeneration, which may be beneficial to minimize culture-induced genetic changes (Fuentes *et al*., 2000 cited in Hussien *et al*., 2006). Induction of somatic embryos from immature leaf cultures of tef without callus phase was reported and highest number of somatic embryos were induced by using 1-5 mg/l dicamba (Frew Mekbib *et al*., 1997). Likyelesh Gugsa (2005) also reported induction of direct somatic embryos from pollinated pistilate and embryo cultures of tef using 2-3 mg/l 2,4-D where as devoid of auxin for zygotic and embryo rescue in which she reported more than 90 % plant regeneration from tef and its wild relatives. Somatic embryogenesis was better achieved in auxins (2,4-D) containing medium and the highest somatic embryo formation for varieties DZ-01-196 and DZ-Cr-37 were achieved when the concentrations of 2,4-D were in the ranges of 2.2-3.3 mg/l. In the present study, somatic embryos were induced via direct somatic embryogenesis using 0-2 mg/l 2,4-D. Eight interspecific crosses between domesticated and wild relatives of tef were carried out and their florets were cultured in six different 2,4-D concentrations. Somatic embryos were induced only from the floret cultures of two F_1 hybrids. However, the floret cultures of other six F_1 hybrids did not induce somatic embryos. This might be due to

mechanical damage of the explants during artificial hybridization, the embryos might be aborted at early stages of development and other factors. Floret cultures from F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri induced somatic embryos in all 2,4-D concentrations except in the control and 2 mg/l 2,4-D. However, floret cultures of F_1 hybrids of *E. pilosa* (30-5)*DZ-Cr-37 induced somatic embryos only in 0.1 mg/l 2,4-D. The florate cultures of F_1 hybrids of *E. pilosa* (30-5)*Kaye Murri induced the highest percentage (46.7 %) of somatic embryos in 1 mg/l 2,4-D followed by 0.5 mg/l 2,4-D (20 %). The present study was different from the embryo rescue work of Likyelesh Gugsa (2005) in the type and composition of the media and types of explants used. The highest percentage of somatic embryos was induced in a 2,4-D concentration within the range of auxins used in the work of Frew Mekbib *et al*. (1997). The study was also in agreement with the induction of calli from immature zygotic embryo cultures of bread wheat. Maximum calli were induced in 1 mg/l 2,4-D (Sears and Deckard, 1982).

The overall frequency of embryo formation from hybrid embryo cultures of *Triticum aestivum*Lymus mollis* was 20 % (Jonsson, 1995). Somatic embryos were induced in mature seed cultures of tef in the range of 0-68.3 % (Kebebew Assefa *et al*., 1998). The highest (98%) plant regeneration was reported in somatic embryo culture of tef from immature zygotic embryos (Likyelesh Gugsa 2005). However, the work of this thesis is the first in its kind to achieve high regeneration from F_1 explant of tef and its wild relatives. In ovule cultures of lily, depending on genotypes, 0.1-1.1 % of somatic embryos has been achieved (Chi, 2002). In barely ovule cultures, 28 % of these ovules contained a poorly developed embryo (Töpfer and Steinbiss, 1985). In the present study, from a total of 635 cultured florets of F_1 hybrids, 21 somatic embryos (3.3 %) of F_1 hybrids were obtained and depending on the parental combinations and treatment conditions, 0-46.7 % somatic embryos were observed.

Likyelesh Gugsa *et al*. (1999) reported that 75 % of the F_1 hybrids from the cross of tef and *E. pilosa* (30-5) did not set especially when the female was the wild spp. Hailu Tefera *et al*. (2003) have reported the achievement of interspecific cross between *E. tef* cv. Kaye Murri and *E. pilosa* (30-5) when the wild was served as a pollen donor (male).

Two hundred recombinant inbred lines derived from individual F_2 plants of the cross *E. tef* cv. Kaye Murri and *E. pilosa* (30-5) were developed through single seed descent method (Teklu Yifru and Hailu Tefera, 2005). However, here with the embryo rescue technique, it is shown that all plants were fertile from the crosses where the female parent being the wild species.The achievement of seed settings from the cross of *E. pilosa* (30-5)*DZ-Cr-37 and *E. pilosa* (30-5)*Kaye Murri was the first success in crossing of tef for the first time. In tissue culture works, the achievement of somatic embryos from F_1 hybrids of tef from both crosses was the first work. The study achieved not only fertile progenies but also used to

micropropagate the hybrid seeds and reduce the breeding cycles of tef varieties to be released through conventional breeding.

6.2.3 Regeneration of plantlets from somatic embryos of F_1 hybrids

Regeneration of plantlets from capitulum explants of gerbera was in half MS medium (Kanwar and Kumar, 2008). Somatic embryos of saffron were induced in MS medium without PGR. Matured somatic embryos were transferred into half MS medium without PGR. Plantlets were regenerated on the same medium in three months (Sheibani *et al*., 2007). Similarly, in the present study, somatic embryos were transferred into half MS medium without PGR. Subculturing was essential to initiate primary somatic embryo formation for cv. Fesho and secondary somatic embryo formation for cv.Dz-01-196 in somatic embryos culture of tef (Likyelesh Gugsa, 2005). Thus, in this study, subculturing was done for three times in the same medium.

Jonsson (1995) reported that 96 % of the F_1 hybrid embryos of *Triticum aestivum***Lymus mollis* have developed into hybrid plants (Jonsson, 1995). In the present study, 50-73.5 % of the somatic embryos regenerated into shoots. Separation of somatic embryos into small pieces and subsequent culture in fresh medium increased the number of plantlets per somatic embryo. The capacity of somatic embryos to regenerate into shoot and root and the high number of plantlets per explant depend on the genotypes, culture media composition and the size of the embryo (Likyelesh Gugsa, 2005). Somatic embryos from the crosses of *E. pilosa* (30-5)*DZ-Cr-37 regenerated one shoot per explant. *E. pilosa* (30-5)*Kaye Murri regenerated 1-10 shoots per explant.

6.2.4 Growth conditions of regenerants

Out of 24 plantlets that were transferred into pots, 100 % of the regenerants were survived in the growthroom. Tweny-one plantlets (87.5 %) were survived out of the 24 plantlets transferred in the glasshouse which is very important for future adoption of the technique to facilitate tef improvement. This might be due to potential of tef to resist harsh environmental conditions is relatively high compared to wheat and other celeals. Only 3 plantlets (12.5 %) died in the glasshouse after three days. This may be due to fungal contamination in the growthroom. A similar case has been reported by Mulu Ayele and Helmut (1995). The regenerants of tef transferred into a glasshouse were survived almost 100 % after a week of transplanting. However, Likyelesh Gugsa (2005) reported that one rescued hybrid from the crosses of *E. tef* and *E. paposa* died after transferring to the glasshouse. In the present study, all plantlets grown in the glasshouse were uniform, normal, fertile and somaclonal varations were not observed. Similar works have been reported by Kebebew Assefa *et al*. (1998) from mature tef seed cultures and

Likyelesh Gugsa (2005) from immature zygotic embryo cultures of tef. Utilization of *E. pilosa* (30-5) in an interspecific cross is a useful strategy for broadening the genetic diversity of the existing gene pool in cultivated tef and as expected for an interspecific cross, distribution of phenotypic values in the progeny showed bi-directional transgressive segregants for all traits, except traits concerned about lodging which showed transgressive segregants towards only the *E. pilosa* (30-5) parent only (Hailu Tefera *et al*., 2003). From the conventional breeding it was possible to get hybrids from the crosses of tef and *E. pilosa* (30-5). In the present study, *E. pilosa* (30-5) has contributed useful breeding traits, such as earliness, panicle form, peduncle length, short stature and may be others to the hybrids. Moreover, Kaye Murri may contributed seed color, vasal stock color, panicle length, culm length, and thicker culms and may be other characteristics to the hybrids. At maturity each somatic embryo induced 8-40 plantlets (tillers) and a total of 442 plantlets (tillers) were produced by the rescued F_1 plants.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

- Three types of explants were used in gynogenic cultures of wheat. Ovary culture was taken as the best explant type for induction of embryonic tissues.
- Light and dark culture conditions were used for development of ovaries and induction of embryonic tissues. Light culture was the best culture condition for induction of embryonic tissues for all varieties.
- Developmental stages of the donor plants were deterimined. According to this investigation, stage II was taken as the best stage for induction of embryonic tissues.
- Cold pretreatment at 4 ^{o} C had effect on induction of embryonic tissues and the results revealed that 15 d cold pretreatment was the best pretreatment duration for induction of embryonic tissues.
- Twelve different concentrations and combinations of 2,4-D and KIN were supplemented in MS basal medium and the results have shown that 1 mg/l of each of 2,4-D and KIN was found to be the best combination for induction of embryonic tissues for all varieties.
- Varieties Simba and Yerer gave the highest embryonic tissues than the other two varieties almost in all combinations.
- MS medium was compared from N_6 and B_5 media and 30 g/l of maltose was compared from 30, 60 or 90 g/l of sucrose or maltose for induction of embryonic tissues. The results revealed that MS was the best medium and 30 g/l of maltose was the best sugar type at a given concentration for induction of embryonic tissues.
- January-March, April-June and July-September were the three types of seasons used for induction of embryonic tissues.
- In ovary culture study, 11 different PGR combinations were used for shoot regeneration. Varieties Simba and Yerer induced the highest frequency of shoots at 0.1 mg/l 2,4-D.
- Simba and Yerer were the most responsive varieties for induction of embryonic tissues as well as shoots.
- Shoots were developed in MS medium supplemented with 60 g/l sucrose and 1.5 mg/l GA_3.
- Shoots of variety Yerer were easily differentiated into well developed shoots and roots (full plantlets).
- If crossing is attempted in wheat, between emmer * durum and emmer*bread, the quality of wheat in the country may be improved.

- In embryo rescue cultures, developmental stages of the F_1 hybrids of tef were studied. The results revealed that florets cultured after 6 days of artificial crossing gave better number of somatic embryos.
- Six different concentrations of 2,4-D were supplemented in MS medium as induction medium. The results have shown that 1 mg/l 2,4-D and 0.1 mg/l of 2,4-D were taken as the best 2,4-D concentrations for induction of somatic embryos from the cross of *E. pilosa* (30-5)*Kaye Murri and *E. pilosa* (30-5)*DZ-Cr-37 respectively.
- Half MS medium without PGR was used as regeneration medium.
- All plantlets transferred into pots were survived in the growthroom. Except three plantlets, the rest survived in the glasshouse and grown till maturity.

7.2 Recommendations

- Including growth conditions of the donor plants, different media compositions & different types of pretreatment conditions may contribute better IETs.

- Temperature of the growthroom and glasshouse should be adjusted into different temperature regimes for proper growth of *in vitro* plants of different species.

- If genetic transformation is included after developing a protocol for regeneration of haploid plants, it will be possible to transfer desired characteristic and eliminate some breeding constraints in order to improve Ethiopian wheat genotypes.

- Further investigations will be needed in order to identify the sources of gynogenic ETs from which of the embryo sac cells originated from**.**

- Flow cytometric analysis should be carried out in order to determine the ploidy levels of tef and wheat regenerants.

- Eventhough crossing of tef was a cumbersome task and only a few personels can carry out, appropriate focus and facilities were not given. Hopefully, if crossing problem could be solved, the bottlenecks of tef will be solved in combination with other modern molecular techniques in the near future.

- RAPD, ISSR, AFLP or other molecular analysis should be carried out, in order to identify qualitative & quantitative characteristics introgressed into the hybrids.

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APPENDIX

Appendix 1. The effect of light and dark culture conditions on the percentage of developed ovaries and induced embryonic tissues.

Culture conditions						
Varieties	**Light**			**Dark**		
	Cultured ovaries	% EO±SD	% ET±SD	Cultured ovaries	% EO±SD	% ET±SD
Simba	220	80.0±12.2	3.4±0.9	220	89.0±8.4	0.0±0.0
Galema	120	86.7±5.5	2.8±1.3	100	87.0±5.0	0.0±0.0
Ude	80	82.5±4.3	3.0±1.5	140	89.3±6.8	1.6±1.3
Yerer	80	80.3±11.4	3.7±1.2	120	83.5±8.9	0.0±0.0

Appendix 2. The effect of cold pretreatment duration on induction of embryonic tissues in ovary culture of wheat using all varieties.

Pretreatments												
	0 d		5 d		10 d		15 d		20 d		25 d	
Varieties	ET	%ET±SD	ET	%ET±SD	ET	%ET±SD	ET	%ET±SD	ET	%ET±SD	ET	%ET±SD
Simba	8	6.0±3.5	10	8.3±2.4	12	10.0±4.0	24	20.0±4.0	11	9.2±1.9	7	5.8±1.9
Yerer	10	8.3±4.4	11	9.2±4.2	13	10.8±2.5	20	16.6±4.5	4	3.3±2.4	1	0.8±2.5
Ude	6	5.0±2.9	7	5.8±2.4	7	5.8±2.4	10	8.3±2.4	5	4.2±2.5	2	1.6±2.4
Galema	4	3.3±2.4	8	6.0±2.4	8	6.0±2.4	16	13.3±2.4	2	1.7±3.2	0	0.0±0.0

Appendix 3. The effect of seasons on induction of embryonic tissues from ovary cultures.

Seasons									
	Janaury-March			April-June			July-September		
Varieties	COs	ET	%ET±SD	COs	ET	%ET±SD	COs	ET	%ET±SD
Simba	80	11	13.8±2.2	80	20	25.0±5.0	60	7	11.8±2.4
Galema	60	4	6.6±2.4	60	1	1.7±2.4	60	1	1.7±2.4
Ude	80	4	5.0±0.0	80	5	6.3±4.7	60	7	11.8±2.4
Yerer	60	2	3.3±2.4	80	11	13.8±2.2	60	2	3.3±2.4

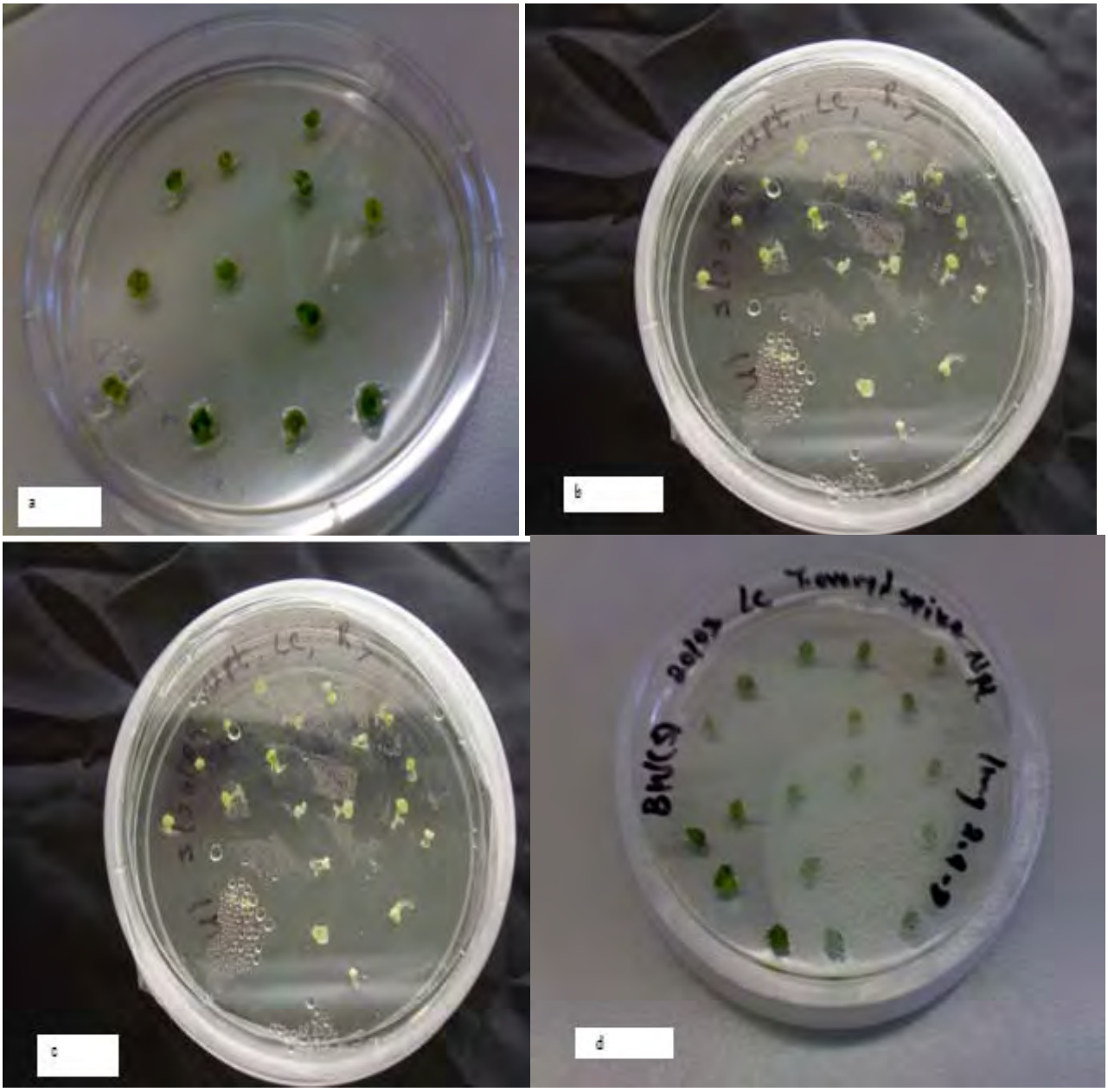
COs =cultured ovaries, ET=Embryonic tissues, SD=Standard deviation

Appendix 4. The effect of different combinations and concentrations of 2, 4-D and KIN for the percentage of induced embryogenic tissues.

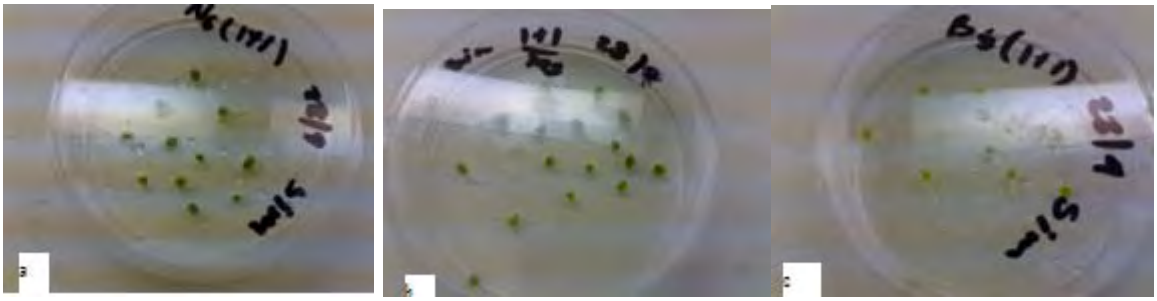
Treatment levels		**Varieties**							
		Simba		Galema		Ude		Yerer	
2,4-D	KIN	IET	% ET±SD	IET	% ET±SD	IET	% ET±SD	IET	% ET±SD
0.5	0.0	8	6.7±2.3	2	1.7±2.3	3	2.5±2.5	12	10.0±0.0
0.5	1.0	8	6.7±2.3	3	2.5±2.5	4	3.3±1.3	14	11.6±2.0
0.5	2.0	11	9.2±3.4	7	5.8±1.9	5	4.2±0.8	14	11.6±4.7
1.0	0.0	24	20.0±4.0	17	14.2±1.9	10	8.3±2.3	20	16.6±2.4
1.0	1.0	32	26.6±4.6	10	8.3±2.9	12	13.3±3.3	42	35.0±9.4
1.0	2.0	17	14.2±5.2	7	5.8±1.8	11	9.2±0.8	19	15.8±3.5
1.5	0.0	14	11.7±4.7	6	5.0±2.8	10	8.3±2.4	17	14.2±4.6
1.5	1.0	10	8.3±3.2	6	5.0±2.8	10	8.3±2.4	13	10.8±1.9
1.5	2.0	9	7.5±2.5	9	7.5±3.8	8	6.7±2.4	8	6.7±2.4
2.0	0.0	7	5.8±1.8	11	9.2±1.9	9	7.5±2.7	7	5.8±2.5
2.0	1.0	6	5.0±1.3	9	7.5±2.5	7	5.8±2.5	6	5.0±2.9
2.0	2.0	9	7.5±2.5	6	5.0±2.8	7	5.8±2.5	4	3.3±1.7

Appendix 5. The effect of different combinations and concentrations of PGRs for differentation of embryogenic tissues.

Varieties													
Treatments		Simba			Yerer			Ude			Galema		
		CET	%RET± SD	TRE	CET	%RET± SD	RET	CET	%RET± SD	TRE	CET	%RET± SD	TRE
0.0 2,4-D	0.0 KIN	39	5.1±1.2	2	22	0.0±0.0	0	17	5.8±2.4	1	14	0.0±0.0	0
0.05mg/l 2,4-D	2.0mg/l KIN	30	3.3±1.9	1	21	23.8±5.5	5	10	0.0±0.0	0	7	0.0±0.0	0
0.05mg/l 2,4-D	3.0 mg/l KIN	35	0.0±0.0	0	24	29.2±4.1	10	9	0.0±0.0	0	12	0.0±0.0	0
0.1 mg/l 2,4-D	0.0 mg/l KIN	29	41.3±13.3	8	24	41.6±14.4	17	8	0.0±0.0	0	10	0.0±0.0	0
0.1 mg/l 2,4-D	1.0 mg/l KIN	29	20.6±10.6	4	15	0.0±0.0	0	6	0.0±0.0	0	9	0.0±0.0	0
0.1 mg/l 2,4-D	2.0 mg/l KIN	26	0.04±0.0	0	17	0.06±0.0	0	7	0.0±0.0	0	11	0.0±0.0	0
0.1 mg/l 2,4-D	2.0 mg/l BAP	24	0.042±0.0	0	22	9.0±5.0	2	18	5.8±2.4	1	9	0.0±0.0	0
0.1 mg/l NAA	0.0 mg/l KIN	19	0.0±0.0	0	23	4.3±1.1	1	9	0.0±0.0	0	7	0.0±0.0	0
0.1 mg/l NAA	1.0 mg/l KIN	25	28.0±11.0	7	27	25.9±9.8	4	17	17.6±6.7	3	15	26.6±13.3	4
0.1 mg/l NAA	2.0 mg/l KIN	24	4.2±2.1	4	16	0.06±0.0	0	7	0.0±0.0	0	7	0.0±0.0	0
0.5 mg/l IAA	1 mg/l BAP	29	0.0±0.0	0	18	0.0±0.0	0	6	0.0±0.0	0	6	0.0±0.0	0



Appendix 6. Comparison of the size of enlarged ovaries of all varieties. a) Variety Galema, b) Variety Ude, c) Variety Yerer, d) Variety Simba.



Appendix 7. Enlared ovaries and induced embryogenic tissues from the three media of variety Simba. a) N6 media, b) MS media, c) B5 media.