III.1 Tall Fescue (*Festuca arundinacea* Schreb.) and Its Hybrids

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1 Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is the predominant cool-season grass in the United States of America, occupying an estimated 12 to 14 million ha (Buckner and Bush 1979). Its primary use is as pasture to support ruminant livestock production.

Tall fescue is adapted throughout Europe and occurs in the Baltic coasts, throughout the Caucasus and in western Siberia extending into China. The species was introduced in North and South America, Australia, Japan, New Zealand, and in south and east Africa. In the USA it is now grown from Florida into Canada and is extensively used as the grass constituent of mixtures in irrigated pastures throughout the western inter-mountain region from southern California to northern Washington (Buckner and Bush 1979).

Many tall fescue plants contain a systemic fungal endophyte, *Epichloe typhina* (Pers.) Tul. (= *Acremonium coenophialum* Morgan-Jones and W. Gams), which is closely associated with pyrrolizidine alkaloids and summer toxicosis symptoms in cattle grazing on the infected plants during periods of high ambient temperatures (Boling et al. 1981). The endophyte survives in plants under a wide range of environmental conditions. Elimination of the endophyte and incorporation of improved forage quality characteristics are desirable goals.

Tall fescue is a wind-pollinated, cross-fertilized species. The most widely grown ecotypes and cultivars of tall fescue are bivalent-forming allohexaploids ($2n = 6x = 42$ chromosomes). Although tall fescue is predominantly self-sterile, some self-fertile plants have been observed. Conventional breeding procedures are a modification of mass selection and recurrent selection methods (Buckner and Bush 1979).

Tall fescue is closely related to a complex group of *Festuca* and *Lolium* species with chromosome numbers ranging from $2n = 14$ to $2n = 70$. The closest relationship are among the four agricultural taxa, *F. pratensis, F. arundinacea, L. perenne,* and *L. multiflorum* (Buckner and Bush 1979). Hybridization of tall fescue with *Lolium* and *Festuca* species offers new sources of germplasm from which superior cultivars may be developed. Chromosome pairing and reassortment of chromosomes and genes in hybrids and their amphiploids result in a

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recombination of specific characters and a new range of genetic variation. Therefore, hybridization may be used as a method of breeding when desirable characters lacking in a species are present in related species or genera.

Intergeneric and interspecific hybrid derivatives of *Lolium* and *Festuca* species that are meiotically stable and fertile have been obtained at the $2n = 42$ chromosome level. Amphiploids of annual and perennial ryegrass × tall fescue ($2n = 56$) and of tall × giant fescue ($2n$ = ca. 56 and 84) hybrids have been obtained with varying degrees of fertility and meiotic stability. Difficulty has been experienced in obtaining meiotically stable, true-breeding colchicine-induced amphiploids. As a consequence, tissue culture methodology is being evaluated (see also Chap. III.2, this Vol.).

2 In Vitro Approaches

Genetic improvement of tall fescue is a slow and tedious process because the plants are generally self-sterile and the pollen is wind-borne. This combination results in difficulty in making controlled crosses, and the need for much greenhouse and field plot space to evaluate large numbers of plants. Consequently, tissue culture approaches were initiated as possible means to accelerate forage grass improvement. The goals were: (a) to develop methodology for establishing callus cultures from superior plants of field-grown tall fescue and fescue hybrids, to induce modification during callus culture, and to regenerate plants from the callus; and (b) to obtain haploid plants from heterozygous field-grown tall fescue plants, to evaluate the haploid plants for forage characteristics under field conditions, to double chromosome numbers (to gain fertility) in superior haploid plants, and to evaluate the doubled haploids under controlled and field environments. The potential value of the tall fescue haploids and their doubled derivatives is the more rapid development of lines with superior forage and/or turf characteristics.

Progress in in vitro callus culture and plant regeneration in monocots has advanced rapidly in recent years, and callus cultures and regenerated plants have been obtained from aseptic seeds and seedlings of a number of cereals (Cheng and Smith 1975; Green and Phillips 1975; Cummings et al. 1976; see also Bajaj 1986) and forage grasses (Ahloowalia 1975; Conger and Carabia 1978; Dale 1980; Bajaj and Dhanju 1981; Bajaj et al. 1981). Also callus and some haploid plants have been obtained from excised anthers of barley (Clapham 1973), rice (Onoo 1975) and wheat (Ouyang et al. 1973; Schaeffer et al. 1979).

However, prior to our work (Kasperbauer et al. 1979, 1980), little was done toward establishment of callus culture from field-grown forage grass plants that had proven superior qualities under field conditions.

The research with tall fescue and its hybrids developed as an extension of our work with tobacco. In tobacco we were able to culture haploid plants directly from anthers of heterozygous plants, grow the haploid plants under various stress conditions, identify individual haploid plants with superior chemical and/or physical characteristics under the stress environment, regenerate fertile doubled

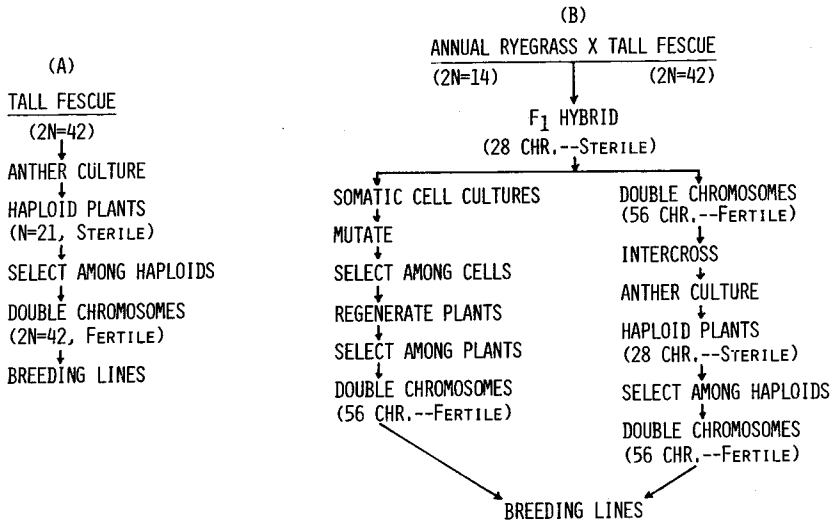

Fig. 1. Working models for micropropagation of tall fescue and annual ryegrass × tall fascue hybrids

haploid plants from "aged" leaf tissue excised from the selected haploid plants, and to evaluate selfed progeny of the doubled haploids under laboratory or field conditions (Kasperbauer and Collins 1972; Collins et al. 1974). Similar objectives were considered desirable for tall fescue and its hybrids.

Working models for micropropagation of tall fescue and the annual ryegrass × tall fescue hybrids are shown in Fig. 1. Both schemes begin with field-grown plants that have exhibited desirable plant characteristics such as winter survival and forage quality factors. Many field-grown plants contained the fungal endophyte, *E. typhina,* which resulted in some contaminated anthers and somatic explant, these were discarded. Nevertheless, we used field-grown plants from the onset in developing our methodology because field plot evaluation was to be an important part of the overall approach.

2.1 Plant Materials and Sterilization

Field-grown plants were collected for somatic explants and for anthers when the panicles showed about 2 or 3 cm above the flag leaf. The plants were cut near the soil surface and immediately placed in flasks of tap water, covered with plastic bags to minimize desiccation, and stored in darkness at 5 °C until explants were taken. Surface sterilization and preparation for transfer to culture medium was done with flame-sterilized instruments in a laminar-flow transfer hood. No attempt was made to remove any microbial contaminant from within the tissue because such procedures might also damage the plant cells.

Explants for somatic tissue cultures were taken from leaf midveins and from the lower end of rapidly elongating internodes and peduncles. Leaf and stem segments about 3 cm long were excised and surface sterilized. The segments were

dipped into 70% ethyl alcohol for a few seconds, washed for 6 min in a 0.8% sodium hypochlorite solution containing a few drops a laboratory detergent, and rinsed in sterile water. After surface sterilization, midveins were excised from the leaf tissue, and the internodes and peduncles were divided into segments that were already elongated and those that were still elongating. The rapidly elongating segments contained may dividing and recently divided cells. Segments about 2–3 mm long were placed on the appropriate culture medium, in sterile, disposable plastic Petric dishes.

For anther culture, panicles were trimmed to remove spikelets with anthers earlier or later than the uninucleate stage. The panicles with remaining spikelets were washed for 5 min in a 0.3% sodium hypochlorite solution containing a few drops of laboratory detergent. The panicles were then rinsed three times in sterile water and left in the final rinse water for a few minutes until individual anthers or panicle segments were excised and placed on appropriate culture medium in sterile plastic Petri dishes.

2.2 Media Composition

The basic medium was modified from Murashige and Skoog (1962) and Linsmaier and Skoog (1965). Inorganic stock solutions and medium contents are shown in Table 1. Agar, sucrose and growth regulator levels differed among various experiments. All components were added to the appropriate amount of sterile water, heated on a stir-hot plate to melt the agar, adjusted to pH 5.7 with 2N NaOH, and autoclaved at 121 °C for 15 min. About 50 ml of autoclaved medium was added to each 100×20-mm sterile plastic Petri dish. The medium was stored for at least 1 day before receiving explants or callus tissue. After placing the tissue, Petri dish covers were taped into position and the dishes were stacked about ten high and covered with transparent plastic bags to maintain humidity. The culture chamber was illuminated continuously from one side by cool-white fluorescent lamps at about 80 $\mu W/cm^2$ between 300 and 800 nm. Aluminium foil was used to cover Petri dishes when darkness was a treatment. Except for medium composition, the same general procedures for media preparation were used for tissue culture, plant regeneration and rooting of plantlets.

2.3 Somatic Callus Cultures

Our initial attempts to induct callus and/or culture plants directly from leaf segments and excised immature anthers of tall fescue or from the annual ryegrass×tall fescue hybrids were totally unsuccessful on a wide range of culture media formulations, preculture temperature treatments, and light and temperature culture environments. Methods that were used to establish callus from immature anthers and regenerate plants from the callus of rice (Onoo 1975) and wheat (Ouyang et al. 1973) were not successful with our tall fescue. Thus, we faced two possibilities: (a) the medium was not appropriate for fescue and its hybrids, or (b) the plant materials were not capable of continued cell divisions and estab-

Table 1. Media used for micropropagation of tall fescue and its hybrids [Modified from Murashige and Skoog (1962) and Linsmaier and Skoog (1965)]

Stock solutions[a]			Culture medium[b]	
Code	Constituent	g/l	ml stock/l medium	mg constituent/ l medium
Basic ingredients of all media				
A	EDTA	0.80	40	32.0
	$Fe_2(SO_4)_3$	0.38		15.2
B	NH_4NO_3	82.50	20	1650
	KNO_3	95.00		1900
C	H_3BO_3	1.24	5	6.2
	KH_2PO_4	34.00		170.0
	KI	0.166		0.83
	$Na_2MoO_4 \cdot 2H_2O$	0.050		0.25
	$CoCl_2 \cdot 6H_2O$	0.005		0.025
D	$MgSO_4 \cdot 7H_2O$	74.00	5	370
	$MnSO_4 \cdot H_2O$	4.46		22.3
	$ZnSO_4 \cdot 7H_2O$	1.72		8.6
	$CuSO_4 \cdot 5H_2O$	0.005		0.025
E	$CaCl_2 \cdot 2H_2O$	88.00	5	440
F	Thiamine·HCl	0.02	5	0.1
	Nicotinic acid	0.10		0.5
	Pyridoxine·HCl	0.10		0.5
	Glycine	0.40		2.0
	myo-inositol			100
(Somatic callus establishment and maintenance, and anther-panicle culture)				
	2,4-D	0.2	10	2 mg
	kin	0.2	0–1	0.0–0.2 mg
	Agar			6 g
	Sucrose			20 g
(Plant regeneration from callus)				
	2,4-D	0.2	1.25	0.25 mg
	kin	0.2	0–1	0.0–0.2 mg
	Agar			6 g
	Sucrose			20 g
(Rooting of regenerated shoots)				
	Agar			5 g
	Sucrose			10 g

[a] Bring components to volume with glass-distilled water and store in a refrigerator. Because of the small amount of 2,4-D and kin used, only 100 or 250 ml of stock solution are prepared. 2,4-D is first dissolved in a few drops of ethyl alcohol, then added slowly to the glass-distilled water.

[b] Add stock solutions and all weighed components (inositol, agar and sucrose) to glass-distilled water, then bring to final volume with glass-distilled water, heat on stir-hotplate until agar melts, adjust to pH 5.7 with 2N NaOH, autoclave at 121 °C for 15 min, cool and pour culture vessels before agar solidifies.

Table 2. Callus formation on stem and leaf explants of annual ryegrass × tall fescue F_1 hybrids cultured on initiation medium supplemented with various levels of 2,4-D and kin (Kasperbauer et al. 1979)

Medium supplement		Explant source		
2,4-D mg/l of medium	kin	Callus formation[a]		
		Leaf vein	Internode tissue	
			Elongating	Elongated
0.0	0.0	–	–	–
0.2	0.0	–	–	–
0.5	0.0	–	±	–
0.5	0.2	–	+	–
1.0	0.0	–	+ +	–
1.0	0.2	–	+ +	–
2.0	0.0	–	+ + +	–
2.0	0.2	–	+ +	–
4.0	0.0	–	+ +	–
4.0	0.2	–	+ +	–

[a] – Indicates no callus formation. +, + +, and + + + indicate callus formation and relative amount.

lishment of callus or plantlets under the conditions used. The first possibility was evaluated by culturing somatic tissue of various ages and origins. Results with the annual ryegrass × tall fescue hybrid are summarized in Table 2. Explants from leaf and stem segments that had already differentiated did not develop callus or plantlets on any of the medium combinations tested. In contrast, the soft young tissue from the lower ends of rapidly elongating internodes developed callus on some of the medium combinations (Table 2) in light or in darkness (Kasperbauer et al. 1979). Also, culture of somatic segments as small in size as tall fescue anthers was less successful than culture with larger explants of the same type of tissue. The results indicated that failure of excised tall fascue anthers to form callus or to regenerate plants may have been due to the small mass of cells capable of division, and possibly the situation could be corrected by using nurse tissue with the anthers. Failure of differentiated stem and leaf tissue to form callus (Table 2) indicated that differentiated tissue of the same plant might provide a suitable tissue mass to serve as nurse tissue for anther culture (Kasperbauer et al. 1980). Ideally, the material would function as nurse tissue but would not be capable of callus formation or plant regeneration.

Callus establishment from young somatic tissue was successful on the basic medium (Table 1) supplemented with 2 to 4 mg of 2,4-D (2,4-dichlorophenoxyacetic acid) per liter with or without kin. Callus formation occurred first at the cut end of the explants as semicompact cream-colored masses. The calli continued to grow well on medium with 2 mg of 2,4-D per liter when subcultured at 3- to 4-week intervals.

2.4 Plantlet Regeneration from Callus

Many leafy shoots developed from calli within a month after they were transferred to medium with decreased 2,4-D content, with or without kin (Table 3). Culture on medium with 2 mg of 2,4-D per liter resulted in rapid callus growth with very little shoot formation. Conversely, decrease of the 2,4-D content of the medium from 2 to 0.25 mg/l resulted in decreased callus growth and increased shoot regeneration. Exclusion of 2,4-D from the medium resulted in even less callus growth and a slight increase in shoot formation. However, elimination of the 2,4-D during shoot formation also favored rooting which caused difficulty in separating the shoots without excessive damage. The individual shoots were ex-

Table 3. Effect of 2,4-D and kin levels on callus growth and shoot formation from established callus. (Kasperbauer 1986)

Medium supplement		Response	
2,4-D (mg/l)	kin (mg/l)	Callus (amount)	Shoots (amount)
2.0	0.2	+ + + [a]	±
2.0	0.0	+ + +	±
0.25	0.2	+	+ + +
0.25	0.0	+	+ + +

[a] ±, +, and + + + indicate relative amounts.

Fig. 2. Annual ryegrass × tall fescue plants that were regenerated from callus cultures of line 38–9, undergoing field evaluation

cised from the calli and transferred to rooting medium without either 2,4-D or kin, and with reduced agar and sucrose contents (Table 1) when they were about 1 cm tall. Roots were formed on the shoots in about 10 to 15 days. After roots formed, the plantlets were transferred to individual containers of sterile potting soil under high humidity at about 21 ± 1 °C. Light from cool-white fluorescent lamps was about 6.5 mW/cm^2 between 300 and 800 nm during plant establishment. Plants were kept on 12-h photoperiods of cool-white fluorescent light to encourage tillering. When they reached adequate size, regenerated plants were transferred to a greenhouse or to field nurseries for evaluation. A plot of regenerated plants of the annual ryegrass × tall fescue line 38-9 is shown in Fig. 2. Most of the plants appeared to be identical, but a low percentage of them had narrow leaves. All flowered at about the same time and were as winter-hardy as the parent hybrid plant.

2.5 Effects of Subculture Number and Duration on Regenerated Plants

Many normal shoots with the same chromosome number as the parent annual ryegrass × tall fescue F_1 hybrid plant were regenerated from callus when transferred to regeneration medium (Table 1) after two subcultures at 3- to 4-week intervals on maintenance medium. This approach could be useful to increase numbers of plants to test various F_1 hybrids after preliminary screening. The procedure could also be useful in detailed evaluation of a genotype under various growth enviroments or in physiological studies in which many plants of the same genotype need to be harvested at different developmental stages (i.e., this method of cloning would be more efficient than propagation by tillers).

Five or more subcultures at the standard 3- to 4-week intervals on maintenance medium before transfer to shoot regeneration medium resulted in fewer regenerated shoots, and in a higher proportion of regenerated shoots that varied phenotypically from the parent plant (Kasperbauer et al. 1979). Low percentages of the regenerated plants had narrow or wide leaves, while the majority very closely resembled the parent plant. Phenotypic variations observed among the regenerated plants suggest that somatic tissue might also be cultured under other stress conditions such as toxins, analogs, etc., to identify those most suitable for regeneration of plants with modified characteristics.

Two standard and one extended subculture of callus of the F_1 hybrid on maintenance medium before transfer to regeneration medium resulted in development of some plants with increased chromosome numbers (Kasperbauer et al. 1979). With tobacco, doubled-haploid shoots were regenerated directly from midveins excised from fully expanded ("aged") leaves of haploid plants while nearly all shoots regenerated from very young leaves were haploids (Kasperbauer and Collins 1972). Since explants from fescue leaves did not form callus or shoots (Table 2), we established callus from "young" internode tissue and subcultured it twice at 3- to 4-week intervals followed by a third subculture of 8 weeks during which the callus began to senesce. The "aged" callus was then cultured on regeneration medium and produced some plants with increased ploidy (Kasperbauer et al. 1979). The experiment showed that endomitosis could occur in callus

tissue and that this might be a useful method to double chromosome numbers in annual ryegrass × tall fescue F_1 hybrids. Recently, we have regenerated doubled haploid plants from aged callus cultured from stem tissue of cytologically-verified haploid tall fescue plants (Kasperbauer and Eizenga 1985).

2.6 Anther Culture

Anthers of field-grown tall fescue plants (2n = 42) were cultured in two ways: (a) individually as excised anthers, or (b) attached to a piece of panicle tissue which was intended to serve as nurse tissue. Since tall fescue tissue that had already differentiated did not produce callus (Table 2), we hypothesized that panicle tissue with all regions of cell division removed, except those in the immature anthers, might be a suitable nurse tissue for anther culture (Kasperbauer et al. 1980).

As discussed earlier in this paper, no calli or plantlets were obtained from excised anthers of any of the fescue varieties and breeding lines tested on a range of media and culture conditions.

Surface-sterilized panicle segments about 2.5 cm in length and containing spikelets with anthers in the uninucleate stage were cultured on the basic medium (Table 1) with 2 mg of 2,4-D and 20 g of sucrose (2%) per liter. Spikelets with anthers earlier or later than the uninucleate stage were removed and discarded as discussed earlier in this paper. Previous work by other investigators showed that more callus was obtained with 9 or 10% than with 2 or 3% sucrose in the medium (Clapham 1973; Wilson 1977). However, plants regenerated from the calli cultured on high sucrose medium included many albinos and abnormal plants (Wilson 1977). Therefore, we used the low level (2%) of sucrose and the level of 2,4-D (2 mg/l) that produced callus proliferation from young somatic explants (Table 2). Changes in the anthers and adjacent tissue became visually detectable after a week of culture; however, many of the panicle explants contained the endophyte, *E. typhina,* which became obvious in about 10 days. Thus, many of the anther-panicle cultures were discarded. Anther-panicle explants from a non-contaminated tall fescue plant continued to change. A loose proliferation of cells became apparent at about 5 weeks of culture. The mass grew rapidly and plantlets were detectable at about 7 weeks in regions not in contact with the medium. There were no albino plantlets, and more than 30 green plantlets originating from different areas of the cell proliferation were transferred to rooting medium and then to sterile potting soil, as described earlier for rooting of regenerated shoots. Twenty-three of the plants survived the transfer and grew rapidly. Twenty-two of them had the haploid chromosome number, n = 21. Somatic metaphase cells of the other plant had 39 chromosomes (Kasperbauer et al. 1980).

After obtaining the haploids we decided to concentrate on evaluating their usefulness in a forage improvement program rather than to continue refinement of methodology for production of more haploids. The haploid plants have been cloned and grown in replicated field plots to evaluate them for forage characteristics. There were pronounced differences among the various haploid lines indicating that they originated from a number of different microspores. Differences occurred among the various haploid lines in winter survival, the relative amounts

of leaf and panicle growth, and some chemical characteristics related to forage quality. Attempts to double chromosome numbers via colchicine treatment were unsuccessful. The "aged" callus approach has resulted in regeneration of some doubled haploids (Kasperbauer and Eizenga 1985), and these will be used to compare characteristics of haploids with those of their doubled haploids when they are grown together in replicated plots. Theoretically, the doubled haploid should have the same characteristics as the haploid plant from which it was cultured, except that the doubled haploid should be fertile. If this is true for tall fescue, haploids will provide a rapid means to identify specific characteristics which would then be present in the fertile doubled haploid which, in turn, could then be used in the breeding program. Further evaluation of the usefulness of haploids and their derivatives involves cytological investigations and attempts to transfer desirable characteristics from doubled haploids to conventionally derived lines.

3 Conclusions and Prospects

Micropropagation of tall fescue and its hybrids offers a possible alternative to the relatively slow conventional procedures for development of forage grass lines with improved agronomic and chemical characteristics. Methodology was developed to establish and maintain callus cultures from somatic tissue, induce change during culture, and to generate plants from the callus. This approach with somatic tissue may be useful to select at the cellular level under stress conditions followed by regeneration of plants from the selected cells.

Green haploid plants were produced from anthers of tall fescue using a nurse tissue approach. A range of phenotypes occurred among the 22 cytologically verified haploid plants. Thus, culture of haploid plants from microspores of a heterozygous fescue plant offers the possibility of rapidly identifying plants with desirable characteristics. Doubling chromosome numbers via aged callus established from the haploid many accelerate development of relatively pure lines for use in the breeding program. Stability of plants regenerated via tissue cultures still needs to be compared with that of plants derived by colchicine treatment.

Our priority was to first evaluate the 22 haploids for potential use in the forage improvement program rather than extend the work on methodology to produce haploids. Considerable work remains to be done on eliminating endophytic contaminants that interfere with culture of androgenic haploids from field-grown plants, and with refinement of the haploid culture procedures.

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