Chapter 4

PLANT REGENERATION AND EVALUATION

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I. INTRODUCTION

Genetic improvement of tall fescue (*Festuca arundinacea*) by conventional plant breeding is very slow because the species is wind-pollinated and generally self-sterile. Because of these factors, it is not possible to rapidly develop inbreds to concentrate desired genes for use in development of new hybrids or cultivars. Therefore, development and use of unconventional methods utilizing cell and tissue culture offer a possible approach to speed up improvement of tall fescue and its hybrids. This chapter will concentrate on development and evaluation of plant regeneration procedures, and on evaluation of somatic tissue-derived plants. Much of the discussion is based on the research of the author and Dr. R. C. Buckner that began in the mid 1970s.

Working models were developed for potential use of cell and tissue culture to speed improvement of tall fescue and an intergeneric annual ryegrass (*Lolium multiflorum*) × tall fescue hybrid (Figure 1). The first callus cultures were established in 1975. Plant regeneration procedures were soon developed by applying principles already known to function in initiation of shoots from tobacco (*Nicotiana tabacum*) callus. That is, tobacco callus will initiate leafy shoots if the auxin/cytokinin ratio is decreased in the culture medium. Since the tall fescue and the hybrid callus cultures did not require cytokinin in the medium, we reasoned that fescue callus was autonomous for cytokinin. If true, we hypothesized that reduction of the auxin (2,4-D) concentration should result in shoot regeneration. It did. Field plot evaluations of somatic tissue-derived plants began in April, 1976, in Dr. Buckner's fescue nursery near Lexington, KY. The experimental approach was subsequently expanded to development and potential use of haploids and doubled haploids in tall fescue improvement, as discussed in Chapters 5 and 6 of this book.

II. PLANT REGENERATION

The ability to regenerate plants from genetically altered cells is critical in development of potentially useful biotechnological approaches to plant genetic improvement. Also, regeneration of numerous genetically identical plants (clones) from potentially superior plants derived from cell cultures (or from conventionally derived plants) is an important part of the fescue germplasm evaluation process. For complete evaluation of potentially useful genotypes, it would be desirable to have enough genetically identical plants to allow simultaneous evaluation for a number of characteristics such as chromosome pairing, forage (and turf) productivity and quality, disease resistance, and tolerance to cold, drought, or heat stress.

Tillering is the natural form of vegetative propagation (cloning) in tall fescue. Therefore, a basic understanding of the tillering process and its regulation should provide some insight for development of methods for plant regeneration from cell and tissue cultures.

WORKING MODELS FOR USE OF CELL AND TISSUE CULTURES

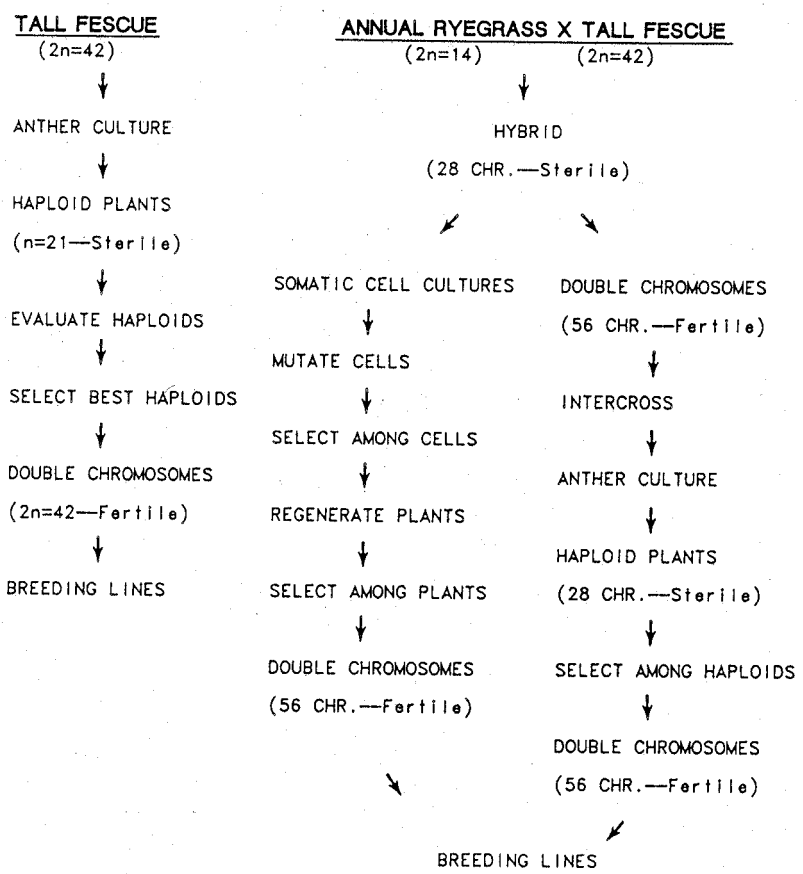

FIGURE 1. Working model for use of cell and tissue culture to speed up improvement of tall fescue and annual ryegrass × tall fescue hybrids. (Adapted from Kasperbauer, M. J., Buckner, R. C., and Bush, L. P., *Crop. Sci.*, 19, 458, 1979.)

A. Tillering

In nature, tall fescue flowers during the long days of spring and early summer, sets seed, goes through "summer dormancy", and then develops tillers during the shortening days of autumn. It is important to realize that tillering in a grass plant is analogous to branching in a broadleaf plant such as soybean (*Glycine max*). That is, tillers develop from axillary buds. Thus, the probability is extremely high that all tillers from the same plant are genetically identical.

The amount of tillering is influenced by the genetics of the plant and the environment under which it is growing. Certain environmental factors are sensed by the plant and various genes are activated or repressed to modify plant development to favor plant survival in the sensed environment.[1] Day length (photoperiod) and the population density of competing plants are major contributors to the environmental aspects. Nutritional status of the plant also contributes to the abundance of tillers, or continued development of tillers after they are initiated.[2]

A tall fescue plant is able to sense decreasing day lengths and initiate vegetative reproduction of itself via tillers. The plant is also able to sense the number and nearness of competing plants, and to autoregulate the amount of tillering. For example, an isolated plant will develop more tillers than will a genetically identical one growing among many other plants, even if all other environmental factors remain constant.

The ability of a plant to sense nearness of competing plants, even before mutual shading occurs, is dependent upon the light absorption and reflection characteristics of leaves. Each green leaf absorbs most of the visible light and reflects or transmits most of the far-red light that it receives. Therefore, the number and nearness of competing leaves influences the amount of reflected far-red light and the ratio of far-red relative to red light received by a plant.[3] The photoequilibrium status of the phytochrome system within the plant is influenced by the far-red/red light ratio.[4] The phytochrome system regulates many developmental processes,[5] such as tillering.[6] A low far-red/red light ratio results in increased tillering, and vice versa. The amount of reflected far-red and the far-red/red light ratio are low when seedlings are small and/or far apart. Under such conditions, the relatively low far-red/red ratio (acting through the phytochrome system) promotes initiation of new tillers. Then, as the tillers increase in number and size, they reflect more far-red light and the far-red/red ratio increases to the point that it stops further tillering. This natural regulatory mechanism warrants further discussion as a useful procedure and as a background in development of *in vitro* cell culture and plant regeneration procedures.

Limited controlled environment study of the environmental influence on tillering of tall fescue has shown that tillering is enhanced under moderately cool, 12 h (relative to longer) photoperiods of bright light that end with a low ratio of far-red relative to red light.[7] Although in-depth controlled-environment studies of tillering have not been published for tall fescue and its hybrids, very relevant studies of tillering have been reported for winter wheat (*Triticum aestivum*).[6] Since results of preliminary unpublished studies with tall fescue were very similar to those conducted with winter wheat, results obtained with wheat are presented in this chapter.

As shown in Table 1, the number of tillers per plant was much higher on wide-spaced field-grown seedlings. Also, the number of tillers per plant at maturity was less than in the seedling stage within each environment. These results are consistent with the concepts discussed above.

TABLE 1
Tillers per Wheat Plant Grown in Wide, Intermediate, and Close (30, 120, and 300 Seedlings per Square Meter) Plantings in a Field

	Plant spacing		
Plant stage	Wide	Intermediate	Close
Seedling	14.0	—	2.9
Mature	9.8	3.5	1.9

Adapted from Kasperbauer, M. J. and Karlen, D. L., *Physiol. Plant.*, 66, 162, 1986.

TABLE 2
Photon Ratios of Far-Red Relative to Red Light Received by Wheat Seedlings Grown in Wide, Intermediate, and Close-Spaced Field Populations (Measurements Taken in Late Afternoon)

Direct sunlight (control)	Plant spacing		
	Wide	Intermediate	Close
0.91	1.15	1.44	1.72

From Kasperbauer, M. J. and Karlen, D. L., *Physiol. Plant.*, 66, 161, 1986.

The photon ratios of far-red/red light received by seedlings in different population densities are shown in Table 2. The ratio was higher in close- than in wide-spaced plants. These readings were all made in sun flecks (i.e., not in shade). Therefore, it is of significance that even the ratio in the wide-spaced population is appreciably higher than that in direct sunlight, away from the plants. It is relevant that earlier studies of plant developmental effects of various wavelengths of light showed that seedlings are very sensitive to even subtle changes in the far-red/red light ratio.[8]

A controlled-environment experiment was conducted with winter wheat seedlings starting a few days after emergence. They received brief exposure to red or far-red at the end of the daily photosynthetic period, and this clearly influenced plant development. Exposure to red (a low far-red/red ratio) resulted in development of more tillers, shorter leaves, and a larger root system than developed on similar plants that received far-red (a high far-red/red ratio) at the end of the daily photosynthetic period (Table 3). These results were consistent with the field responses (Tables 1 and 2). The response to a brief

TABLE 3
Effects of Phytochrome on Tillering, Leaf Length, and Shoot/Root Biomass Ratios of Wheat Seedlings in Controlled Environment

Plant character	Light at end of 12-h day		
	R	FR	FR + R
Tillers/plant (no.)	8.7 ± 0.3	5.4 ± 0.3	8.1 ± 0.2
Leaf length (mm)	425 ± 5	460 ± 5	420 ± 5
Shoot/root biomass (ratio)	1.09	1.36	1.10

Note: Red (R) and far-red (FR) light treatments were for 5 min at the end of daily 12-h photosynthetic periods for 20 consecutive days. The FR + R treatment consisted of 5 min of FR followed immediately by 5 min of R to test photoreversibility of the control system.

From Kasperbauer, M. J. and Karlen, D. L., *Physiol. Plant.*, 66, 162, 1986.

exposure to red immediately following far-red is evidence of photoreversibility of the light receptor and demonstrates that the receptor is phytochrome.

It is clearly evident that tillering is regulated by environmental sensors within the plant that measure day length and spectral distribution of light, and then regulate developmental responses to favor survival of the plant. That is, tillering would lead to production of more potential flowering stems which would be favorable up to a point; however, the plant can stop further tillering when the sensing system determines that continued tiller formation would be counterproductive.

The important points in this discussion are that day length (photoperiod) signals the plant to produce tillers to clone itself, and the far-red/red ratio (as is influenced by the amount of reflected far-red light as a measure of size, number, and nearness of competing plants) acts through the phytochrome system in autoregulation of amount of tillering. It is apparent that the phytochrome system functions as a variable switch that constantly monitors the far-red/red ratio (as a measure of competition) and regulates tillering as well as other developmental processes.

Since natural tillering results in vegetative propagation of a relatively few (although genetically identical) plants, it would be reasonable to draw on this information to develop cell and/or tissue culture methods that could result in large numbers of genetically identical plants from a superior field-grown plant. Such methods would be useful whether the plant was derived through conventional plant breeding or through biotechnological approaches.

B. Plant Regeneration from Cell and Tissue Cultures

A number of experiments designed to regenerate plants from somatic cells were conducted with tall fescue and an intergeneric annual ryegrass × tall

fescue hybrid that was developed by Dr. Buckner. The hybrid was an attempt to combine forage qualities of ryegrass and the persistence of fescue.[9] The F_1 hybrid has 7 chromosomes from annual ryegrass and 21 chromosomes from tall fescue. The 28-chromosome hybrid is not fertile. Chromosome doubling is necessary to obtain fertility. Cell and tissue culture approaches were introduced as part of the research and development program.

Since many of the pioneering studies of cell and tissue culture were done with tobacco, experience with that species served as a basis for development of tissue preparation methodology, media formulation, and physical environments selected for initial studies with tall fescue and its hybrid. In addition to the development of basic nutrient media for cell cultures,[10,11] some of the early studies demonstrated that tobacco callus tissue could be induced to form shoots or roots by changing the auxin/cytokinin ratio in the nutrient medium. Other early research utilized tobacco tissue cultures to demonstrate that undifferentiated callus contained phytochrome and that the ratio of far-red/red light received by the callus acted through the phytochrome system within the cells to influence metabolic processes.[12,13] Callus that received red light for 5 min (alternated with 23 h and 55 min of darkness) per day accumulated significantly more dry matter during a 14-d treatment period.[12] The far-red/red photoreversible response in undifferentiated callus cultures was reported in 1966.[12] It was the first evidence that phytochrome is present in and can regulate metabolic processes in undifferentiated callus on artificial nutrient medium. The fact that phytochrome is present in dividing and recently divided cultured cells is relevant in selecting a light source, or darkness, for use during cell and tissue cultures and during plant regeneration from those cultures. Photodestructability of various components of the medium should also be considered when selecting a light regime for tissue culture and for plant regeneration from cell and/or tissue cultures. Some of these apparently subtle differences brought about by exposure to light may be major factors in success or failure in plant regeneration from cell and tissue cultures. After considering the foregoing factors, a number of experiments were conducted with tall fescue and the intergeneric hybrid to develop cell and tissue culture and plant regeneration methods analogous to those already proven successful for tobacco.

1. Explant Source

a. Embryos

Immature embryos and/or embryos from germinating seeds are among the most convenient materials from which to establish cultures. Embryos are relatively easy to surface decontaminate, establish callus, and regenerate plants. Embryo-derived cultures are useful for development of medium requirements and physical environment conditions for a given species. The plants regenerated from embryo-derived tissue cultures have a high probability (relative to leaf- or stem-derived callus) of being genetic clones because of the nature of the explant source. However, a major disadvantage of cloning a highly

heterozygous species, such as tall fescue, from embryo cultures is that there is a very high probability that each embryo is genetically different from all other embryos. Therefore, there is no assurance that a randomly selected embryo would contain desired basic forage and/or turf characteristics and cold, heat, or drought tolerance. To obtain that and other relevant information, it would be desirable to evaluate the plants under field conditions to eliminate undesirable genotypes before establishment of cell and/or tissue cultures. For example, incorporation of improved forage (or turf) characteristics via cell or tissue culture would be much more useful if incorporated into a plant that is field hardy. Therefore, establishment of aseptic tissue cultures from field-grown plants is a desirable approach. However, it is much more difficult than establishment from embryos.

b. Field-Grown Plants

A number of experiments were conducted with explants from field-grown plants to compare explant source, medium additives, physical environment, and timing of subcultures. Critical factors and rationale for various approaches are discussed below.

Field-grown plants allow preselection of source plants for growth, development, and survival characteristics before establishing the cell cultures. Of course, a field-grown plant has a much greater probability of microbial contamination on the leaf or stem surface as well as within the tissue. This fact increases the importance of surface decontamination of the explants. It is important to kill the microbial contaminants without killing the critical plant cells. A slight modification of procedures used for field-grown tobacco[14,15] was successful for field-grown tall fescue and its hybrids.

Vigorously growing plants were selected during rapid spring growth when panicles began to emerge. Leaf, stem, and peduncle explants were selected and cut into 2- to 5-cm segments. These were surface decontaminated by washing for about 6 min in 15% laundry bleach and 85% water (i.e., about 0.8% sodium hypochlorite in water). All subsequent steps were done in a sterile transfer hood. The decontamination process was followed by three brief rinses in sterile water to remove the remaining sodium hypochlorite from the tissues. The selected somatic explants were then cut to 2- to 5-mm segments, trimmed of extraneous tissue, and placed on appropriate culture medium. The explants were cut and trimmed under sterile water to avoid drying at the cut surfaces before placement on the medium.

2. Culture Media

Although a number of medium formulations have been used for cell and tissue cultures of various species, we used the same basic medium for tall fescue and its hybrids as used for tobacco tissue cultures. The medium formulation, prepared as six stock solutions (Table 4), is based on our modification[13,15] of Murashige and Skoog[10] and Linsmaier and Skoog.[11] The

TABLE 4
Composition of Basic Fescue Culture Media

Constituent	Stock solution[a] Code	Stock solution[a] g/l	Culture medium[b] ml/stock l medium	Culture medium[b] mg constituent/ l medium
Medium I. Somatic Callus Establishment and Maintenance, and First-Stage of Anther-Panicle Culture				
EDTA	A	0.80	40	32.0
$Fe_2(SO_4)_3$		0.38		15.2
NH_4NO_3	B	82.50	20	1650
KNO_3		95.00		1900
H_3BO_3	C	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
$MgSO_4 \cdot 7H_2O$	D	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
$CaCl_2 \cdot 2H_2O$	E	88.00	5	440
Thiamine · HCl	F	0.02	5	0.1
Nicotinic acid		0.10		0.5
Pyridoxine · HCl		0.10		0.5
Glycine		0.40		2.0
2,4-D	—	0.20	10	2.0
Sucrose	—	—	—	20,000
Agar	—	—	—	6,000
myo-Inositol	—	—	—	100

Medium II. Plant Regeneration from Callus, and Second-Stage of Anther-Panicle Culture

Composition is identical with Medium I, except that 1.25 ml of 2,4-D stock is used giving a final concentration of 0.25 mg/l

Medium III. Rooting

Composition is identical with Medium I, except that no 2,4-D is used, agar is reduced to 5,000 mg/l, and all other components are half strength

[a] Bring components to volume with glass distilled water. Because of the small amount of 2,4-D used, a smaller amount of stock solution is prepared. The 2,4-D is first dissolved in a few drops of ethyl alcohol, then added slowly to the glass-distilled water.

TABLE 4 (continued)
Composition of Basic Fescue Culture Media

[b] Add stock solutions and all weighed components (sucrose, agar and inositol) 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 2 *N* NaOH. Autoclave at 121°C for 15 min. Cool and pour culture vessels before agar solidifies.

Adapted from Kasperbauer, M. J. and Wilson, H. M., in *Nicotiana: Procedures for Experimental Use* (USDA Tech. Bull. 1586), Durbin, R. D., Ed., U.S. Department of Agriculture, Washington, DC, 1979, chap. 5.

present author considered this to be a logical approach because whole plants of both species have the same basic nutrient requirements, and the objective was to accomplish with tall fescue and its hybrids what was already accomplished with tobacco (i.e., establish and maintain callus cultures, culture haploids, and regenerate doubled haploids from somatic tissue excised from haploid plants).[15] The only significant difference between media used for fescue and tobacco was the amount of auxin and cytokinin in the medium.

3. Tissue Source

Explants from leaf veins, elongated stems, and soft tissue from the base of peduncles and internodes were cultured on the basic medium supplemented with a range of auxin and cytokinin concentrations. As shown in Table 5, no callus formed on leaf and stem segments that had already differentiated on the source plant. This was in contrast to tobacco in which cell cultures were easily established from fully expanded leaves and from stem pith.[15] Many of the explants from fully expanded fescue stem tissue from field-grown plants also contained a slow-growing (in culture) endophyte identified as *Epichloe typhina* (now known as *Acremonium coenophialum*). An important finding was that callus formed only from the very young tissue at the base of rapidly elongating peduncles and internodes when cultured on medium containing 2 to 20 mg of 2,4-D per liter of medium. A concentration of 2 mg of 2,4-D per liter was subsequently used as the standard level for callus establishment and maintenance because this level was effective and because higher levels appeared to have undesirable side effects. An added bonus was that the callus established from the youngest tissue was not contaminated with the endophyte. This is analogous with earlier research that resulted in disease-free plants through meristem culture.[16] The results shown in Table 5 provided several important items of information: (1) the medium formulation allowed callus establishment from explants that had many dividing or recently divided cells, (2) callus did not form from expanded leaf or stem explants (this became a very important factor for culture of tall fescue haploids using panicle tissue as a nurse culture that was not capable of callus formation or plant regen-

TABLE 5
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, NAA, and Kinetin

Medium supplement (mg/l)			Explant source and callus formation[a]		
				Internode tissue	
2,4-D	NAA	Kinetin	Leaf vein	Elongating	Elongated
0.0	0.0	0.0	–	–	–
0.0	0.1	0.2	–	–	–
0.0	0.1	2.0	–	–	–
0.0	2.0	0.2	–	–	–
0.2	0.0	0.0	–	–	–
0.5	0.0	0.0	–	±	–
0.5	0.0	0.2	–	+	–
1.0	0.0	0.0	–	+ +	–
1.0	0.0	0.2	–	+ +	–
2.0	0.0	0.0	–	+ + +	–
2.0	0.0	0.2	–	+ +	–
4.0	0.0	0.0	–	+ +	–
4.0	0.0	0.2	–	+ +	–

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

From Kasperbauer, M. J., Buckner, R. C., and Bush, L. P., *Crop Sci.*, 19, 458, 1979.

eration, as discussed in Chapter 5 of this book), and (3) the auxin requirement was much higher, and the cytokinin requirement was lower for fescue than for tobacco. In fact, cytokinin was not needed in the medium for callus establishment, maintenance, or plant regeneration.

4. Physical Environment

Because tissue culture studies with tobacco had shown that the cells contain phytochrome and exhibit a growth response to the ratio of far-red relative to red light,[12,13] fescue explants from young peduncle and stem tissue were cultured under various lighting regimes. As shown in Table 6, there was no dramatic difference in percentages of explants that formed callus in light vs. darkness. Although some differences were apparent among genetic lines, all formed some callus. Awareness that callusing occurred in both light and darkness allowed flexibility in development of protocols, such as maintenance of cultures in a dark cabinet (with brief daily illuminations during inspections). In subsequent experiments, it was determined that low-intensity

TABLE 6
Callus Formation on Explants from Elongating Internodes and Peduncles of Annual Ryegrass × Tall Fescue F_1 Hybrids and Parental Lines at 25 ± 1°C in Light or Darkness on Media Containing 2 or 4 mg of 2,4-D per Liter

	Callus formation on explants			
	Light		Darkness	
Source of explants	No. of calli/ no. of explants	%	No. of calli/ no. of explants	%
Parental lines				
Annual ryegrass	—	—	7/12	58
Tall fescue	—	—	8/13	62
F_1 hybrid lines				
13—1	14/18	78	12/19	63
20—1	9/21	43	16/27	59
20—5	30/49	61	20/29	69
38—9	7/32	22	5/24	21
38—15	13/15	87	19/22	86
38—19	13/25	52	12/21	57
38—20	8/22	36	18/23	78
58—12	9/17	53	10/18	56
58—30	22/37	59	18/30	60
58—32	18/22	82	15/20	75

From Kasperbauer, M. J., Buckner, R. C., And Bush, L. P., *Crop Sci.*, 19, 458, 1979.

light from cool white fluorescent lamps was useful during plant regeneration from callus because it kept the regenerating plants from becoming etiolated.

5. Plant Regeneration

Numerous plants were regenerated from tall fescue and from annual ryegrass × tall fescue hybrid callus tissue by decreasing the 2,4-D concentration of the medium to 0.00 or 0.25 mg/l (Table 7). Preliminary experiments using the basic medium with the decreased 2,4-D level showed that plant regeneration occurred in darkness or in light within 3 to 4 weeks. Plants were routinely regenerated from callus under low-intensity light from cool white fluorescent lamps in order to avoid etiolation. Cool white fluorescent lamps were used because they emit a low ratio of far-red relative to red light, which acts through the phytochrome system to suppress shoot elongation and favor rooting (see Tables 1 to 3).

Complete removal of 2,4-D from the regeneration medium resulted in no new callus formation and many regenerated plants with abundant roots. This combination resulted in tangled roots and much difficulty in separating the plants. Consequently, many plants were damaged during separation and transfer to starting medium. On the other hand, the 0.25 mg level of 2,4-D resulted

TABLE 7
Callus and Plant Regeneration Response of Tall Fescue Callus Cultures to 2,4-D Level after Establishment and Maintenance on Basic Medium with 2 mg 2,4-D per Liter

2,4-D conc.	Response[a]	
	Callus	Plantlets
(mg/l)		
4.0	+ + +	−
2.0	+ + +	±
0.25	+	+ + +
0.0	−	+ + +

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

Adapted from Kasperbauer, M. J., Buckner, R. C., and Bush, L. P., *Crop Sci.*, 19, 458, 1979.

in formation of some new callus and regeneration of many plants, with very few roots. These plants were easy to separate when they were about 1 cm tall. Very few were damaged, and they rooted quickly when placed on a rooting medium (half-strength basic medium [Table 4] with 5 g of agar per liter and no 2,4-D). Rooting and plant establishment were done in a 21 ± 1°C growth chamber with light from cool white fluorescent lamps. When the roots were about 1 cm long, individual plants were coded and root tips were collected and processed for cytological evaluation (see Chapter 7 of this book). The plants were returned to sterile rooting medium until new roots were about 1 to 2 cm long, after which the plants were transferred to sterile potting soil. The newly potted plants were covered with transparent plastic to maintain high humidity for 3 d. The starting chamber was controlled at 21 ± 1°C and illuminated for 12 h/d with cool white fluorescent light at about 200 μmol $m^{-2} s^{-1}$. After the 3rd d, the plastic cover was removed for a few hours each day to condition the plants to lower humidity. The plastic cover was completely removed after another 3 d. The light level was gradually brought up to that of full sunlight, and plants were transferred to a greenhouse. After establishment of the regenerated plants under greenhouse conditions, more than 1200 of them were taken to field plots for evaluation in conjunction with Dr. Buckner's tall fescue improvement program.

Tillers were taken from some of the field-tested plants and grown in a controlled environment selected to produce numerous new tillers (i.e., 12-h days from cool white fluorescent lamps [low far-red/red ratio] at 21 ± 1°C). The tillers were used as a source of somatic tissue for cytological evaluation.

III. EVALUATION OF REGENERATED PLANTS

Successful regeneration of plants from cell and tissue cultures is an important step. However, thorough evaluation is needed before they are usable in a plant improvement program.

A. Visual

Early visual evaluation of newly regenerated shoots can identify obvious albinos, which occur in greater frequency when the regeneration medium contains a high concentration of sucrose.[17,18] Other abnormalities, such as highly abnormal leaves and failure to develop roots, are also identifiable during the rooting phase, before plants are transplanted to potting soil.

B. Cytological

Cytological evaluations (discussed in detail by Springer in Chapter 7 of this book) should involve determination of chromosome numbers, pairing, and other possible abnormalities. Determination of chromosome numbers is an important step in determining potential usefulness of the regenerated plants. After the initial screening for chromosome number and evaluation of growth characteristics, more detailed analyses of chromosome pairing should be done on the plants that are considered to be the best candidates for incorporation into the breeding program (see Chapter 7 for further discussion).

C. Field

After preliminary visual and cytological screening to reduce the numbers, the regenerated plants should be grown under field conditions to evaluate summer heat tolerance, winter cold hardiness, and growth characteristics that may determine usefulness of the genotype. Forage and/or turf characteristics should be determined for each candidate plant. The author's experience with field evaluation of regenerated plants is discussed below.

In order to have sufficient replicated samples for the field studies, we first grew the regenerated plants in pots under a controlled environment that caused profuse tillering. The vegetatively propagated plants were then coded and planted in replicated field plots for evaluation.

The first group of about 1200 regenerated plants were started in pots in a greenhouse and then moved to field plots in April 1976. They were evaluated over a 2-year period. Field evaluations during subsequent years were done with smaller numbers of preselected plants and for various durations, depending on the objectives.

The plants shown in Figure 2 were photographed in late spring, following their first winter in field plots. Nearly all plants survived the winter, and many of them appeared to be identical. A key point became apparent during the evaluation. The number and duration of subcultures prior to plant regeneration was important. Plants that were regenerated from callus after two 3-

FIGURE 2. Annual ryegrass × tall fescue plants that were regenerated from callus cultures.

to 4-week subcultures on the maintenance medium (2 mg of 2,4-D per liter) were compared with plants regenerated after five 3- to 4-week subcultures and with others regenerated after one or two 3- to 4-week subcultures and one 8-week subculture before plant regeneration. Plant regeneration proficiency and the general characteristics of the regenerated plants obtained by these three procedures are discussed below. The general pattern of results provided the basis for subsequent research to develop procedures for successful culture of tall fescue haploids (Chapter 5 of this book) and regeneration of doubled haploids from cytologically verified haploids (Chapter 6 of this book).

1. Plants Regenerated after Two Brief Subcultures

Many normal-appearing shoots developed and appeared to be clonal reproductions of the original plants. Root tip cytology showed that all of the regenerated plants also had the same chromosome number as the original plants. Thus, under the conditions used, callus could be established from young stem tissue from tall fescue and the annual ryegrass × tall fescue hybrid, and normal plants could be regenerated from the callus after two brief subculture periods on maintenance medium. This procedure showed promise for rapid clonal increase of a plant that exhibits outstanding characteristics in

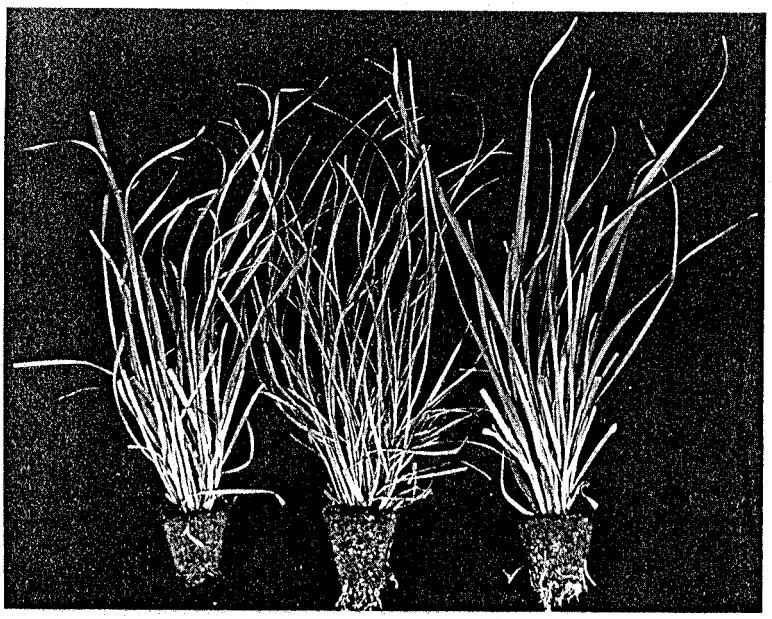

FIGURE 3. Normal-, narrow-, and wide-leaved plants regenerated from the fifth subculture of callus established from the same young stem explant from annual ryegrass × tall fescue F_1 hybrid line 58-12. (From Kasperbauer, M. J., Buckner, R. C., and Bush, L. P., *Crop Sci.*, 19, 458, 1979.)

a field evaluation. Such rapidly produced clones developed from selected plants could be very useful to increase plant numbers for use in detailed physiological studies in which the same genotype must be evaluated under a number of different environments, or be sampled at different developmental stages.

2. *Plants Regenerated after Five Brief Subcultures*

Calli that went through five successive 3- to 4-week subcultures before being placed on the plant regeneration medium developed fewer shoots per mass of callus than those that went through only two 3- to 4-week subcultures before plant regeneration. Also, a higher percentage of the plants regenerated from the "five-subculture" series were phenotypically variant from the original source plant. In one study with tissue excised from an annual ryegrass × tall fescue F_1 hybrid plant, a small percentage of the regenerated plants had very fine leaves, and an equally small percentage had wider than normal leaves (see Figure 3) even though plants of all three phenotypes had the chromosome number of the source plant. These variants were identified in 1975 and have been vegetatively propagated through tillers until the present

time (1989) for possible further use in some aspect of forage or turf improvement. The plants have been evaluated for forage characteristics and for summer and winter hardiness in Kentucky and South Carolina.

Subsequent experiments with tall fescue also showed that as the number of subcultures increased, the ability to regenerate plants decreased and the percentage of phenotypic variants (also called somaclonal variants) increased. Recent examination of a number of regenerated tall fescue plants for root characteristics showed that some of the regenerated lines had distinctly different root systems, even though shoots appeared the same and chromosome numbers did not differ. We are now (1989) researching the possible use of cell and tissue culture to develop plants with superior root systems. For example, roots with the ability to penetrate a high bulk-density subsoil layer would be a major advantage on soils with low natural fertility and low water-holding capacity in the surface layer. These experiments have shown that the percentage of phenotypic variants could be increased by increasing the number of subcultures before regenerating plants. Conversely, a large number of apparently identical plants could be obtained by regenerating plants after only one or two brief subcultures.

3. Plants Regenerated after One Brief and One Extended Subculture

This approach was a preliminary attempt to mimic some tobacco culture responses and regenerate plants with higher ploidy than the source plant. With tobacco, it is possible to excise midvein tissue from a fully expanded leaf and to regenerate plants with the doubled chromosome number directly from the leaf tissue. A modified approach was taken with fescue because earlier attempts to culture plants, or to establish callus, from fully expanded ryegrass × fescue leaf or stem tissue were unsuccessful (Table 5). We reasoned that a possible alternative with fescue was to establish callus from young stem tissue (Table 5) and, after increasing the mass of callus during one or two subcultures, to "age" the callus during an extended subculture before regenerating plants. We hypothesized that the same chain of events that took place to induce endomitotic divisions followed by regeneration of plants from "aged" tissue taken from fully expanded tobacco leaves[14,15] might be duplicated by "aging" the fescue callus on maintenance medium before regenerating plants. The first experiments were done with callus derived from the annual ryegrass × tall fescue F_1 hybrid. These plants had 28 chromosomes (7 from the ryegrass and 21 from the fescue parent). The F_1 hybrid (like a tobacco haploid) was infertile, and plants with the doubled number of chromosomes might be fertile (like a tobacco doubled haploid). The initial attempts with "aged" callus from the F_1 hybrid resulted in 111 regenerated plants that were grown as individual entries in field evaluation plots. Cytological analyses were not completed before the field evaluation because of a scheduling problem. All plants survived the first winter. Although most of the regenerated plants had the same general phenotypic characteristics as the F_1 source plant,

11 of them displayed visible amphiploid characteristics[9] such as larger stems and wider leaves. Root tip analyses of the 11 putative amphiploids showed that 9 of them had the doubled number of chromosomes, 1 had the F_1 number, and the other one was not countable. This preliminary experiment demonstrated that endomitotic divisions could be stimulated in such callus and that plants with the increased chromosome number could be regenerated. This information provided the basis for later experiments in which tall fescue doubled haploids were cultured from callus derived from haploids (Chapter 6 of this book). The fact that not all of the regenerated plants with visually detected amphiploid characteristics had the doubled chromosome number emphasizes the importance of using cytological procedures as part of the overall evaluation of regenerated plants that are derived through various biotechnological methods.

IV. SUMMARY

Observation of tillering responses of fescue to various environmental conditions, and experience with tobacco tissue culture and plant regeneration, provided model systems for development of tall fescue tissue culture and plant regeneration. Although callus cultures are more easily established from immature embryos than from field-grown fescue plants, field-grown plants are prescreened for a number of characteristics such as winter or summer hardiness. Thus, this chapter concentrated on establishment and maintenance of callus cultures from field-grown fescue, plant regeneration, and various facets of evaluation. It also attempted to show how successful use of fescue cell and tissue cultures can contribute to more rapid isolation of pure lines that can speed up genetic improvement of this important wind-pollinated, self-infertile, polyploid perennial grass species.

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