Detection of Somaclonal Variation in Tissue Culture Regenerants of Tall Fescue

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ABSTRACT

Clones of tall fescue (*Festuca arundinacea* Schreb.) regenerated from tissue culture are required for examining interactions between the endophyte, *Acremonium coenophialum* Morgan-Jones and Gams, and its host. Somaclonal variation is common among regenerated plants, and could limit the utility of this technology to study the interactions of tall fescue and its endophyte. Various methods are used to detect somaclonal variation, but there is little agreement as to the relative advantages of each. Therefore, the objectives of this study were to determine (i) whether somaclonal variation exists among tall fescue plants regenerated from somatic embryos, and (ii) whether pollen viability, yield, morphological traits, or phenological development give consistent estimates of somaclonal variation. Seven regenerants from non-infected tall fescue genotype PDN2 and a non-regenerated PDN2 control were planted in the field in five replications. Plants were screened for somaclonal variation by phenological development, vegetative and flag leaf width/length ratios, total biomass, seed yield, and pollen viability over 2 yr. Although regeneration protocols were designed to minimize somaclonal variation, somaclonal variation of quantitative traits was observed. Total biomass and seed yield of regenerants, used together, were most consistent in detecting somaclonal variation. When using tissue culture to insert endophytes into tall fescue, we found multiple regenerants need to be infected with each isolate of endophyte to ascertain whether differences among response variables are due to endophyte or somaclonal variation.

SEVERAL STRATEGIES have been used to study interactions between tall fescue and its fungal endophyte. One method is to infect clones of a common tall fescue genotype with different endophyte isolates to assess endophyte-mediated changes in the plant (Hill et al., 1991b). Infection of clones of a common tall fescue genotype with different endophyte isolates necessitates the use of plant tissue culture techniques (Kearney et al., 1991) because the endophyte will not infect mature tall fescue plants (Bacon and Siegel, 1988). A concern of using tissue culture to study tall fescue/endophyte interactions is the possibility that somaclonal variation may confound the effect of an endophyte, making it difficult to assess the endophyte's contribution to plant responses.

Concomitant with concerns that somaclonal variation exists, is the ability to document its existence. Traits that have been evaluated to detect somaclonal variation in plants include pollen viability, plant morphology, phenology, and yield. Eizenga and Dahleen (1990) used pollen viability to identify somaclonal variation in tall fescue, while others have used leaf width/length ratios (Cummings et al., 1976), plant height (Hanna et al., 1984), heading and flowering dates, or seed yield (Stephens et al., 1991) as traits for other species.

There is no consensus as to which traits consistently detect somaclonal variation. Possible reasons for the lack of consensus are that (i) the traits chosen by individual investigators are those which are easiest to measure, (ii) traits may be affected by genotype × environmental interactions, and (iii) selected traits are not always based on a variety of unrelated characters (De Klerk, 1990). Therefore, the objectives of this study were to determine (i) whether quantitative traits were affected by somaclonal variation in tall fescue plants regenerated from somatic embryos; and (ii) examine if pollen viability, yield, morphological traits, or phenological development are the most consistent variables to use when screening for somaclonal variation.

MATERIALS AND METHODS

Plants regenerated by somatic embryogenesis (Kearney et al., 1991) were used in this experiment. Explants were taken from meristematic regions at the base of elongating tillers from the endophyte-free tall fescue genotype PDN2. The explants were surface-sterilized in a commercial bleach solution (10.5 g L^{-1} $NaClO_3$) for 30 sec and in 700 mL L^{-1} EtOH solution for 3 min. Explants were rinsed three times with sterile water, cut into 2.5-cm lengths, and placed on a basal medium to induce callus formation. The embryonic medium consisted of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 30 g L^{-1} sucrose, 30 μM 2,4-D, and 20 g L^{-1} Gelrite (Gibson Laboratories, Inc., Lexington, KY) as a solidifying agent. The pH of the medium was adjusted to 5.8 prior to autoclaving. Cultures were placed in a growth room with 30 μM m^{-2} sec^{-1} photon flux density provided by cool white fluorescent tubes; daylength was maintained at 23 h and temperature at 26 ± 1°C. After 28 d on embryonic medium, calli were transferred to auxin-free basal medium to permit growth and development of somatic embryos. Germinating embryos were excised from the calli and placed in GA-7 containers (Magenta Corp. Chicago, IL) with auxin-free basal medium with a sucrose level of 6 g L^{-1}. After 28 d, plants were transferred to soil and allowed to harden. The plants were transferred to the greenhouse and placed in 4-L pots containing a 1:1:1 mixture of Cecil sandy clay loam soil (Typic Kanhapludult, mixed, mesic, thermic)/peat/perlite.

Of 77 endophyte-free regenerated plants (Kearney et al., 1991), regenerants R12, R16, R19, R22, R30, R37, and R40 of tall fescue genotype PDN2 were randomly selected for this experiment. Individual tillers were taken from each regenerated plant, cut to 5-cm lengths and screened for a uniform mass of 2 g fresh weight. They were also screened so each contained two visible tiller primordia at the basal node. Fifteen tillers were selected from each regenerant and transplanted into 10-cm pots containing the soil mixture used previously, and grown in the greenhouse for 28 d. The same was done for tillers from the non-regenerated parent plant PDN2. Five plants of each regenerant were selected only if they had developed three tillers with two expanded leaves on each tiller. This was conducted to assure that all plant materials were physiologically and developmentally uniform, thereby reducing variability within and among regenerants that could be attributed to the physiological condition of the vegetative propagules.

On 1 Oct. 1990, the regenerants were transferred to the

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Abbreviations: FWL, flag leaf width/length ratio; VWL, vegetative leaf width/length ratio; PCSAS, statistical analysis systems for personal computers.

Table 1. Significance values for response variables used to estimate somaclonal variation of PDN2 regenerants for Year (Y), regenerants (R), and Y × R.

Trait	Y	R	Y × R
Panicle emergence	*	ns	ns
Flowering date	**	ns	ns
Seed maturity	*	ns	ns
Pollen viability	**	ns	ns
Total biomass	**	**	ns
Seed weight	**	**	ns
VWL†	*	*	**
FWL‡	ns	*	*

*, ** Significant at the $P < 0.05$ and $P < 0.01$ level levels, respectively.
† Vegetative leaf width/length ratio.
‡ Flag leaf width/length ratio.

Table 2. Vegetative and flag leaf width/length ratios among PDN2 tall fescue regenerants over 2 yr.

	Vegetative leaf		Flag leaf	
Plant	1991	1992	1991	1992
R12	0.398	0.194	0.392	0.398
R16	0.490	0.234	0.442	0.518
R19	0.472	0.230	0.446	0.484
R22	0.400	0.186	0.396	0.426
R30	0.432	0.224	0.404	0.496
R37	0.400	0.204	0.408	0.476
R40	0.386	0.218	0.422	0.448
PDN2	0.346	0.200	0.362	0.352
LSD (0.05)†	0.060	0.023	0.044	0.055

† Least significant difference at the 0.05 level of probability.

field and planted in a Pacolet soil (Typic Kanhapludult, clayey, kaolinitic, thermic) at the UGA Plant Sciences Farm near Watkinsville, GA. The soil had been plowed, disked, and fumigated with methyl bromide on 15 Sept. 1990. One plant of each clone was placed 0.67 m apart in five randomized complete blocks, with each block serving as a replication. At planting, the plot was fertilized with N, P, and K at a rate of 80, 35, and 66 kg ha^{-1}, respectively. Plots were fertilized again on 5 Mar. 1991, and 14 Feb. 1992 with 150, 66, and 125 kg ha^{-1} N, P, and K, respectively.

Phenological development, width/length ratio of vegetative (VWL) and flag (FWL) leaves, pollen viability, total biomass production, and seed yield were measured in an effort to detect somaclonal variation. To measure phenological development, plants were evaluated every other day beginning on 1 April of each year and dates recorded when (i) the first tiller reached panicle emergence from the boot, (ii) the stamens first emerged from a developing panicle, and (iii) physiological maturity of the seed was reached. For the purposes of this experiment, physiological maturity was defined as being the point when the endosperm was solid and retained its shape after being squeezed between the thumb nails. Width/length ratios of vegetative leaves were determined from 15 randomly selected leaves prior to elongation of tillers during each spring. Leaf blade lengths were measured from the collar to the tip of each leaf blade, and the width of the leaf blade measured at its midpoint. To measure flag leaf width/length ratios, the process was repeated on 10 flag leaves from tillers which had fully emerged panicles.

Pollen viability was measured from panicle pieces containing 10 to 15 immature florets. Two anthers were removed from two unopened florets and squashed between a slide and cover slip. Extruded pollen grains were stained with acetocarmine (Eizenga and Dahleen, 1990) and viable pollen determined as the percent which stained red from four microscopic fields viewed from each anther.

Above ground biomass was determined by hand clipping the plants at a height of 7.5 cm above the soil surface. The tissue was dried at 55°C for 72 h and total dry weight recorded. Seed yield was determined by clipping the seedheads after the herbage was dried, and hand-threshing the seed from each plant.

Data were analyzed by analysis of variance with a randomized complete block model using the PROC ANOVA subroutine of the PCSAS computer program (SAS Institute, Cary, NC). Means were separated by the MEANS/LSD statement in the analysis of variance. Variances within regenerants were tested for homogeneity with the Bartlett's test for homogeneity (Little and Hills, 1978, p. 140-150). After confirming homogeneity of variances, we estimated relative somaclonal variation for clones derived from the tissue culture protocol and the vegetative propagation protocol, using the standard deviation among the regenerants and mean standard deviation within the regenerants, respectively.

RESULTS

No differences between regenerants and the control PDN2 were found for dates of panicle emergence, flowering, and seed maturity, or pollen viability. However, differences existed among total biomass, seed yield, VWL, and FWL measurements (Table 1). Of the four traits which varied among regenerants, VWL and FWL showed a year × regenerant interaction, but there was no year × regenerant interaction for total biomass and seed weight.

In 1991, regenerants R12 and R40 were not different from the PDN2 control for VWL, while in 1992 regenerants R12, R22, R37, and R40 were not different from the PDN2 control (Table 2). In 1991, regenerants R12 and R22 were not different from the PDN2 control for FWL, but in 1992, only R12 was not different from the control. In 1991, regenerant R40 was not different for VWL, but differed from the control for FWL, and regenerant R22 was different for VWL, but was not different from the control for FWL. In 1992, regenerants R22, R37, and R40 were different for FWL, but had VWL values that were not different from that of the control.

Regenerants R16, R19, and R22 were not different from PDN2 for total biomass (Table 3). Regenerants R12, R22, R30, and R40 were not different from PDN2 for seed yield. Only R22 was not different from PDN2 when total biomass and seed yield were considered together.

Mean standard deviations within regenerants were approximately 1/3 of the standard deviation among regenerants for VWL, FWL, total biomass, and seed yield, indicating that somaclonal variation was negligible during the vegetative propagation of the regenerant clones (Table 4).

DISCUSSION

Somaclonal variation is a concern whenever plant tissues are cultured in vitro. Regeneration of grass species via somatic embryogenesis is believed to be less subject to somaclonal variation than regeneration via organogenesis, as embryo ontogeny is sensitive to disruption by

Table 3. Means for total biomass and seed yield for PDN2 tall fescue regenerants averaged over 2 yr (1991, 1992).

Plant ID	Total Biomass	Seed Yield
	g plant^{-1}	
R12	418.40	19.28
R16	303.45	16.72
R19	302.76	16.06
R22	315.29	19.48
R30	384.95	22.33
R37	444.88	34.10
R40	431.02	31.64
PDN2	317.46	24.24
LSD (0.05)†	57.77	7.51

† Least significant difference at the 0.05 level of probability.

any abnormality (Ozias-Akins and Vasil, 1988). Even though tall fescue regenerates via somatic embryogenesis, the incidence of somaclonal variation in cultured tall fescue tissues and regenerated plants has been extensively documented (Reed and Conger, 1985; Eizenga, 1987, 1989). The DNA content in tall fescue callus has been found to increase with time in culture (Conger et al., 1980) which affects the extent of somaclonal variation (Kasperbauer et al., 1979; Eizenga and Cornelius, 1991).

In this study, we tried to minimize opportunities for somaclonal variation by minimizing the time in culture as much as possible (Kearney et al., 1991). In previous studies, aneuploidy has been documented to occur in 40 to 60% of regenerated tall fescue plants (Reed and Conger, 1985; Dahleen and Eizenga, 1990). Based on the aneuploidy occurance rate of 40 and 60% in those experiments, we would have expected to find aneuploidy among the seven regenerants at the 0.972 and 0.998 probability level in this experiment, respectively. However, we detected no aneuploidy. Furthermore, the regenerated PDN2 plants expressed no obvious evidence of somaclonal variation. Although the modified regeneration protocols successfully prevented the occurence of gross somaclonal variation, this did not exclude the possibility that more subtle variation was present. Previous greenhouse studies showed no differences among regenerants (Hill et al., 1991b). The current study differs in that the same plants were grown in replicated, spaced plantings under field conditions. Variability was detected within year for VWL and FWL, but this was not consistent over years, suggesting these measurements of somaclonal variation are prone to error. Differences in VWL between 1991 and 1992 may be due to early season environmental conditions or bias in sample selection, while differences in FWL may be due to late season environmental conditions. Alternatively, somaclonal variation could have affected the stability of these traits.

Total biomass and seed yield consistently differed over years, suggesting these estimates of variability are the best of those tested to detected somaclonal variation. Being consistent over years also suggests that testing over multiple years may be unnecessary to detect somaclonal variation by these parameters. However, since no single regenerant varied for both of these traits, both are needed to more accurately assess the presence of somaclonal variation.

The fact that pollen viability was not different from

Table 4. Variability within and among regenerants of tall fescue for response variables that expressed somaclonal variation among the PDN2 regenerants.

	Standard deviation of regenerants	
Response variable	Within	Among
VWL†	0.031	0.097
FWL‡	0.040	0.127
Total biomass	61.3	185.4
Seed yield	8.3	21.1

† Vegetative leaf width/length ratio.
‡ Flag leaf width/length ratio.

that of the control suggests that the somaclonal variation among regenerants was not a result of gross genetic changes in the plants. Eizenga (1987) found that reduced pollen viability was a consequence of aneuploidy in regenerated tall fescue plants. Due to the subtle nature of the variation found, the greenhouse assay previously used (Hill et al., 1991b) was inadequate to detect the variation present. Detection of somaclonal variation in field-grown plants may be due to stresses associated with winter, insects, diseases, drought, or response to natural progressions in day length. All of these factors affect the phenotype (De Battista and Bouton, 1990) and may explain why differences were detected in field-grown but not greenhouse-grown plants.

The use of molecular markers has been advocated as a way to screen for somaclonal variation in the genus *Festuca* (Valles et al., 1993). However, their studies were unable to detect any variation, even when chloroplast DNA fragments were used as probes on albino plants. Therefore, molecular markers may not be a sufficiently sensitive method with which to screen for somaclonal variation unless sufficient probes are used to saturate the entire genome (Isabel et al., 1993).

Phenological development and pollen viability were not different among regenerants. This could be interpreted to mean no somaclonal variation existed, but the variability for leaf morphology, total biomass and seed yield was approximately three times as great among regenerants as it was within vegetative propagules of the regenerants. This suggests that somaclonal variation was present among regenerants, but was not readily detectable.

The lack of readily detectable differences in development does not exclude the possibility that somaclonal variation of a more subtle or quantitative nature is present (Larkin and Scowcroft, 1981). Such variation can be detected by replicated field trials over time. Although somaclonal variation was not a result of gross genetic changes, six of seven regenerants expressed variability even though the plants were regenerated by protocols most likely to minimize the amount of somaclonal variation.

Tissue culture insertion of endophytes into tall fescue has utility for studying interactions between the two mutualistic organisms. Phenotypes of plant/endophyte associations can be modified by specific plant genotype/endophyte isolate interactions (Fletcher, 1993; Hill, 1993). Being able to discern the contribution of each organism for traits such as drought stress (Elmi et al.,

1989), insect and disease resistance (Clay et al., 1985; Clay, 1988), or alkaloid production (Hill et al., 1991a) may enable scientists to develop an understanding of plant/endophyte associations to capitalize on the attributes of each organism. Precautions need to be taken to reduce the amount of somaclonal variation when following this method, even when tissue culture protocols are designed to reduce somaclonal variation.

Previous studies with plants derived from the tissue culture endophyte insertion method have investigated the effects of endophytes and their hosts on ergot alkaloid content (Hill et al., 1991b). In that study, only one plant regenerant was infected with each endophyte isolate, raising concerns about validity of their conclusions based on the possibility that somaclonal variation confounded the endophyte effect. To more appropriately assess how plants or endophytes interact requires tissue culture infection of multiple tall fescue regenerants by a common endophyte. A determination of whether somaclonal variation were a factor in plant responses could then be made by quantitating variability within regenerants containing a common endophyte and comparing it to variability among regenerants with different endophytes treatments.

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