Tissue Culture and Genetic Transformation of Some Turfgrass Genera

Hassan Salehi1* • Mohamadreza Salehi1 • Mariam B. Sticklen2

1 Department of Horticultural Science, College of Agriculture, Shiraz University, Shiraz, Iran
2 Department of Crop and Soil Science, Michigan State University, East Lansing, MI, 48824 USA

Corresponding author: * hsalehi@shirazu.ac.ir

ABSTRACT

Tissue culture and genetic transformation of some turfgrass genera are reviewed. Our recent reports are also included in detail. Four turfgrass genera were used, namely common bermudagrass, Cynodon dactylon (L.) Pers. (California origin); strong creeping red fescue, Festuca rubra L. var. rubra ‘Shadow’; perennial ryegrass, Lolium perenne L. ‘Barbal’; and Kentucky bluegrass, Poa pratensis L. ‘Merion’. After acid treatment and surface sterilization, seeds were cultured on Murashige and Skoog (MS) basal medium supplemented with 40 μM dichlorophenoxyacetic acid (2,4-D) for Cynodon and Poa, 150 μM 2,4-D for Lolium and 200 μM 2,4-D for Festuca. Acid treatments improved both callus production percentage and rate, compared to control. Cynodon had the highest callus production rate and thereafter were Lolium, Poa, and Festuca. Cynodon had the best callus production in light and the other genera in dark conditions. In the second experiment, callus induction and plant regeneration were studied in turfgrass genera used. Mature seeds were surface-sterilized and cultured on MS medium supplemented with 30 to 250 μM 2,4-D for callus induction. Regeneration medium consisted of MS supplemented with 5 to 10 μM 6-benzyladenine (BA). Among the genera, Poa had the highest callus induction percentage regardless of 2,4-D concentration, followed by Cynodon, Lolium and Festuca. Cynodon and Lolium had the highest callus regeneration percentage and overall regeneration rate. Reported transformation studies on the genera used are discussed in the text.

Keywords: Agrobacterium, Biolistic® bombardment, callus, explant, gene transfer, lawn, somatic embryogenesis
Abbreviations: CIP, callus induction percentage; CRP, callus regeneration percentage; ORR, overall regeneration rate

CONTENTS

INTRODUCTION ..................................................................................................................... 25

Turfgrass: definition and culture......................................................................................... 25
Importance of biotechnology and tissue culture studies in turfgrass industry..................... 25
Turfgrass tissue culture ....................................................................................................... 26
Genetic transformation ....................................................................................................... 26

METHODS ........................................................................................................................ 27

Experiment 1: Effects of the seed germination treatments on callus induction in four turfgrass genera.......................................................... 27
Experiment 2: Comparison between callus induction and plant regeneration of turfgrass genera ........................................................................ 27

RESULTS ........................................................................................................................ 28

Experiment 1: Effects of seed germination treatments on callus induction in turfgrass genera ........................................................................ 28
Experiment 2: Comparison between callus induction and plant regeneration of turfgrass genera ........................................................................ 28

DISCUSSION .................................................................................................................... 29

Experiment 1: Effects of seed germination treatments on callus induction in turfgrass genera ........................................................................ 29
Experiment 2: Comparison between callus induction and plant regeneration of turfgrass genera ........................................................................ 30

REFERENCES .................................................................................................................. 30

INTRODUCTION

Turfgrass: definition and culture

Turfgrasses are referred to those grass species suitable to be used as turf, i.e., a covering of vegetation, plus the matted, upper stratum of earth filled with roots and rhizomes (Beard 1973). Less than 50 grass species are used as turf because of their ability to persist under regular mowing (Christians 1998). Moreover, turfgrasses are also used to beautify the earth, enrich our lives, and provide recreation for enjoyment. Turfgrass is maintained on lawns, estates, parks, golf courses, play, and public grounds (Chai and Sticklen 1998). More than 245 warm- and cool-season turfgrass cultivars have been registered since 1946 in the U.S.A. (Lee 1996). These achievements have contributed to the establishment and growth of the turfgrass industry and the development of turfgrass science (Beard 1973).

The turfgrass industry has undergone rapid growth in its attempts to meet the increasing public demands for products and services.

Importance of biotechnology and tissue culture studies in turfgrass industry

Every success in genetic transformation relies significantly on the ability to obtain transformable and regenerable explants through tissue culture (Li and Qu 2004). Some materials are transformable, but not regenerable, thus one can only obtain transgenic calli that not succeed to regenerate
into plants (Bai 2001). To perform efficient genetic transformation of turfgrasses, it is necessary to optimize tissue culture conditions which involve genotypes, explant types, and culture media (Forster and Spangenberg 1999; Bai 2001). Using more diverse genotypes in one tissue culture study may be useful, showing similarities and/or differences in factors such as type of explant and concentrations of plant growth regulators in the medium (Salehi and Khosh-Khui 2005a).

**Application of biotechnology in turfgrass management**

Using genetic engineering as the central component (Boulter 1995), biotechnology provides a dominant tool in the development of desirable turfgrass cultivars tolerant to stresses such as, drought, salt, pests, diseases and herbicides (Chai and Sticklen 1998). Other desirable tasks, include developing turfgrass cultivars with low maintenance requirements such as dwarf or rosette turfgrasses (Xu et al. 1995) or cultivars with delay in flowering time that would result in prolonged vegetative growth (Salehi et al. 2005a), which could reduce the mowing times.

**Turfgrass tissue culture**

In most studies, plant tissue culture techniques have been used to propagate a single cultivar or several cultivars in one species of turfgrass. There are few comparative tissue culture studies made between different turfgrass species or genera. Yoo and Kim (1991) investigated the effects of different plant growth regulators on callus formation and organogenesis of four turfgrass species and genera namely, Japanese or Korean lawngrass, creeping bentgrass, Bermuda grass, and Kentucky bluegrass (Poa pratensis L.). In particular, significant progress has been made in the establishment of tissue culture techniques for Lolium L. sp. and Festuca L. sp. (Forster and Spangenberg 1999). Tissue culture (in vitro manipulation) of monocytes, particularly the cereals and grasses, was initially found to be rather difficult (Bai 2001). This difficulty was overcome largely as a result of using high concentrations of strong auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) or 3,6-dichloro-o-anisic acid (dicamba) and the culture of immature embryos or seeds, leaf base meristems and meristematic segments of young inflorescences (Vasil 1995).

It was noted that callus derived from somatic cells varies in its competence to express totipotency (Bai 2001). In monocots, explants that contain immature, meristematic cells develop a callus that is more likely to express totipotency. In cereals tissue culture plants have been developed from specific regions of the callus via either somatic embryogenesis or organogenesis (Bhaskaran and Smith 1990).

**Culture medium and plant growth regulators**

Generally, high salt nutrient medium, Murashige and Skoog (MS) medium (Murashige and Skoog 1962), with 3 to 12% sucrose and a solidifying agent and high concentrations of 2,4-D or dicamba have been generally used for turfgrass tissue culture (Chai and Sticklen 1998).

The use of low levels of cytokinins, such as N6-benzylaminopurine (BAP), in combination with auxins has been reported to enhance embryogenic callus formation and regeneration ability (Zhang et al. 1991; van der Valk et al. 1995; Chaudhury and Qu 1998). Mature seeds were also used for Lolium perenne L. (Creemers-Molenaar et al. 1988; Wu et al. 2005; Bajaj et al. 2006) and Poa pratensis L. (van der Valk et al. 1995). Mature embryos have been used for Lolium perenne L. (Dalton 1988) and P. pratensis L. (McDonnell and Conger 1984; Stephens et al. 2006). Immature embryos (Gao et al. 2006) and shoot apices (Hu et al. 2006) are used in P. pratensis L. In Cynodon species, most somatic embryos were produced by using young inflorescences (Ahn et al. 1985, 1987, Artunduaga et al. 1988, Chaudhury and Qu 2000; Li and Qu 2002, 2004, Lee et al. 2005; Zhang et al. 2007) or mature seeds (Ahn et al. 1985, 1987; Salehi and Khosh-Khui 2003, 2005a; Salehi et al. 2005b).

**Genetic transformation**

Genetic transformation can accelerate crop improvement. Progress in turfgrass transformation has been made in exploring and optimizing transformation systems that have been used for other grass species and dicots (Chai and Sticklen 1998).

**Biolistic® bombardment**

Microbiolistic delivery systems for turfgrass genetic engineering have achieved success. Transformation by Biolistic® (Bio-Rad Lab. Inc., Hercules, CA) delivery has been reported in all the species used (Table 1).

**Agrobacterium-mediated transformation**

Gene integration patterns are more predictable in this method than other methods such as Biolistic® bombardment (Smith and Hood 1995). Transformants from Agrobacterium-mediated transformation contain genomic insertions by exact and single or low copy number of transgene cassettes (de Block 1993). Single-copy insertions facilitate gene expression because they are less prone to cosuppression or gene silencing effects that are usually found in multicopy insertion events (Vasil 1995).

**Table 1** List of Biolistic®-transformed turfgrass species used and related information.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Transgenes</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common bermudagrass</td>
<td>gus, hpt</td>
<td>Li and Qu 2004</td>
</tr>
<tr>
<td>[Cynodon dactylon (L.) Pers.]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red fescue (Festuca rubra L.)</td>
<td>hpt</td>
<td>Spangenberg et al. 1995a</td>
</tr>
<tr>
<td></td>
<td>nptII</td>
<td>Spangenberg et al. 1998</td>
</tr>
<tr>
<td></td>
<td>gus, hpt</td>
<td>Ahteter and Xu 2000</td>
</tr>
<tr>
<td>Perennial ryegrass</td>
<td>gus, hpt</td>
<td>Cho et al. 2000</td>
</tr>
<tr>
<td>[Lolium perenne L.]</td>
<td>gus, hpt</td>
<td>Van der Maas et al. 1994</td>
</tr>
<tr>
<td></td>
<td>gus, hpt</td>
<td>Spangenberg et al. 1995b</td>
</tr>
<tr>
<td></td>
<td>nptII</td>
<td>Dalton et al. 1999</td>
</tr>
<tr>
<td></td>
<td>nptII, RgMV-CP</td>
<td>Xu et al. 2001</td>
</tr>
<tr>
<td>Kentucky bluegrass</td>
<td>gus, hpt</td>
<td>Petrovskia et al. 2004</td>
</tr>
<tr>
<td>[Poa pratensis L.]</td>
<td>hgt, gus, hpt</td>
<td>Hisano et al. 2004</td>
</tr>
<tr>
<td></td>
<td>gus, hpt</td>
<td>Chen et al. 2005</td>
</tr>
<tr>
<td></td>
<td>bar, gus, hpt</td>
<td>Hu et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gao et al. 2006</td>
</tr>
</tbody>
</table>

Genotypic differences may be related to endogenous hormone levels. Even explants regenerated from a single genotype may not respond identically in culture (Wernickle and Brettell 1982).

**Somatic embryogenesis studies in important turfgrass genera**

Previous tissue culture studies on different genera and species of turfgrasses showed that callus induction and plant regeneration were possible with culturing mature seeds of two cultivars of red fescue (F. rubra L. var. commutate Gaud. ‘Jamestown’) (Torello et al. 1984) and F. rubra L. var. trichophylla Gaud. ‘Dawson’ (Torello et al. 1984; Zaghmout and Torello 1988). Mature seeds were also used for Lolium perenne L. (Creemers-Molenaar et al. 1988; Wu et al. 2005; Bajaj et al. 2006) and Poa pratensis L. (van der Valk et al. 1995). Mature embryos have been used for Lolium perenne L. (Dalton 1988) and P. pratensis L. (McDonnell and Conger 1984; Stephens et al. 2006). Immature embryos (Gao et al. 2006) and shoot apices (Hu et al. 2006) are used in P. pratensis L. In Cynodon species, most somatic embryos were produced by using young inflorescences (Ahn et al. 1985, 1987, Artunduaga et al. 1988, Chaudhury and Qu 2000; Li and Qu 2002, 2004, Lee et al. 2005; Zhang et al. 2007) or mature seeds (Ahn et al. 1985, 1987; Salehi and Khosh-Khui 2003, 2005a; Salehi et al. 2005b).
Another advantage of Agrobacterium-mediated DNA delivery is the minimal exposure of explant material to tissue culture conditions that induce genetic instability (Karp 1993). Although grass and other monocots have been generally considered outside the host range of A. tumefaciens, Agrobacterium-mediated transformation has been used successfully in a few grass species including maize (Gould et al. 1991; Ishida et al. 1996), rice (Chan et al. 1992, 1993; Liu et al. 1992; Park et al. 1996), and wheat (Deng et al. 1988).

Monocotyledons such as grasses may not produce phenolic compounds like acetosyringone (Cheng et al. 2004). Using acetosyringone or other phenolic compounds might be useful for more efficient transformation.

There are a few reports on Agrobacterium-mediated transformation of turfgrass species used (Table 2).

### METHODS

Four important turfgrasses genera commonly used in the world were used in this study. They are briefly described below. All the seeds were purchased from Barenbrug Co., The Netherlands.

A. Perennial ryegrass, Lolium perenne L. ‘Barball’. Perennial ryegrass, a member of Poaceae family, Poaideae subfamily and Festuceae tribe is native to the temperate regions of Asia and North Africa (Darlington and Wylie 1961; Beard 1973). This is a cross-fertilized cool-season turfgrass with high growth rate, moderate wear tolerance, moderate salinity resistance, and low drought resistance (Beard 1973).

B. Kentucky bluegrass, Poa pratensis L. ‘Merion’. Kentucky bluegrass, a member of Poaceae family, Poaideae subfamily and Festuceae tribe is a native of Eurasia (Darlington and Wylie 1961; Beard 1973). This is a cross-pollinated (facultative apomictic) cool-season turfgrass with slower growth rate than Lolium, moderate wear tolerance, moderate drought resistance and poor salinity resistance (Beard 1973).

C. Strong Creeping Red Fescue, Festuca rubra L. var. rubra ‘Shadow’. Red fescue, a member of Poaceae family, Poaideae subfamily and Festuceae tribe is a native of Europe (Darlington and Wylie 1961; Beard 1973). This is a cross-pollinated cool-season turfgrass with good growth rate, high shade tolerance, moderate wear tolerance, good drought resistance, poor salinity resistance and is very susceptible to high temperatures (Beard 1973).

D. Common Bermudagrass, Cynodon dactylon (L.) Pers. (California origin). Common Bermudagrass, a member of Poaceae family, Eragrostioideae sub-family and Chlorideae tribe is a native of eastern Africa (Darlington and Wylie 1961; Beard 1973). This is a cross-pollinated warm-season turfgrass with prostate growth, excellent wear tolerance and excellent drought and salinity resistance (Beard 1973).

### Experiment 1: Effects of the seed germination treatments on callus induction in four turfgrass genera

Four mentioned turfgrass genera were used in this study.

### Sulfuric acid treatments

In this study, based on the previous findings (Salehi and Khosh-Khui 2005b) the following treatments with sulfuric acid were used: Cynodon with 50% sulfuric acid for 20 min, Festuca with 25% acid for 15 min, Lolium with 50% acid for 10 min, and Poa with 50% acid for 15 min, all with their controls. Seeds were kept continuously shaken in cold acid, and they were rinsed three times with distilled water and were then placed under running tap water overnight.

### Culture

Seeds were surface sterilized using 70% ethanol for 1 min followed by 100% laundry bleach (5.25% sodium hypochlorite) for 20 min and then rinsed six times with distilled sterilized water.

Based on preliminary experiments, Murashige and Skoog (1962) (MS) basal medium (Sigma-Aldrich, St. Louis, MO) (Murashige and Skoog 1962) containing 30 g/l sucrose and 8 g/l agar-agar (Merck, Darmstadt, Germany) supplemented with 40 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for Cynodon and Poa, 150 μM 2,4-D for Lolium and 200 μM 2,4-D for Festuca was used for callus induction. Media were autoclaved at 22.04 psi and 121°C for 15 min. After cooling the media to 50°C, filtered, 2,4-D was added. The sterilized media were then divided into 10 cm sterilized plastic Petri dishes. Each treatment consisted of 4 replications and 100 seeds per replicate. Each treatment was repeated at least three times.

### Light/dark or dark treatments

Effects of light/dark conditions were evaluated in each genus with or without acid treatment.

### Environmental conditions

All cultures were kept at 25°C. In light/dark treatments, cultures were placed in a 16/8 h light/dark photoperiod. Light intensity was 140 μmol/m²/s emitted by cool white fluorescent 40 W lamps (Pars Co., Iran).

### Data recording and statistical analysis

After 4 weeks, the number of seeds which induced callus formation was scored. In each genus and treatment, the number of days needed for callus induction to be seen was also recorded. Completely randomized design (CRD) was used for all experiments with factorial arrangement. Data were analyzed using MSTAT-C software (Freed and Eisen-smith 1989). Arcsine transformation was carried out on percentage data. Means were separated using Tukey’s test at the 1% level (P<0.01).

### Experiment 2: Comparison between callus induction and plant regeneration of turfgrass genera

Mature seeds were used in this comparative study.

### Dehulling and surface sterilization

Seeds were dehulled using the following treatments: Cynodon with 50% sulfuric acid for 20 min, Festuca with 25% acid for 15 min, Lolium with 50% acid for 10 min, and Poa with 50% acid for 15 min (Salehi and Khosh-Khui 2005b). Seeds were continuously shaken in cold acid, rinsed at least three times with distilled water, and placed under running tap water overnight. Seeds were surface-sterilized in 70% ethanol for 1 min, followed by 100% laundry bleach for 20 min, and then rinsed six times with sterilized distilled water.

### Culture

MS basal medium containing 30 g/l sucrose and 8 g/l agar-

---

**Table 2: List of Agrobacterium-mediated transformed turfgrass species used and related information.**

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Transgenes</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common bermudagrass</td>
<td>cry1Ac, gus, hpt, nptII</td>
<td>Salehi et al. 2005b</td>
</tr>
<tr>
<td>Cynodon dactylon (L.) Pers.</td>
<td>gus, hpt</td>
<td>Li et al. 2005</td>
</tr>
<tr>
<td>Red fescue (Festuca rubra L.)</td>
<td>-</td>
<td>Wang and Ge 2005</td>
</tr>
<tr>
<td>Perennial ryegrass</td>
<td>bar, gus, OsNHX1</td>
<td>Wu et al. 2005</td>
</tr>
<tr>
<td>Lolium perenne L.</td>
<td>gus, hpt</td>
<td>Bajaj et al. 2006</td>
</tr>
<tr>
<td>Kentucky bluegrass</td>
<td>gus, hpt</td>
<td>Cao et al. 2006</td>
</tr>
</tbody>
</table>
agar (Merrick, Darmstadt, Germany) supplemented with the following growth regulators was used. According to preliminary experiments, 0 (control), 30, 40, 50, 60, 150, 200, and 250 µM 2,4-D was used for callus induction media. Basal MS media containing 30 g/l sucrose and 8 g/l agar-agar supplemented with 0 (control), 5, 7, 10, 12.5, or 15 µM 6-benzyladenine (BA) (Sigma-Aldrich, St. Louis, MO) were used for plant regeneration. The rooting medium was a plant growth regulator-free half-strength MS medium containing 30 g/l sucrose and 8 g/l agar-agar. The pH of each medium was adjusted to 5.8 with 0.1 M NaOH prior to autoclaving at 22.04 psi and 121°C for 20 min. Filter-sterilized medium was adjusted to 5.8 with 0.1 M NaOH prior to autoclaving at 22.04 psi and 121°C for 20 min. Filter-sterilized 2,4-D and BA were added after autoclaving when the medium cooled down to 50°C. The sterilized media were divided into 10-cm sterile plastic Petri dishes. Plantlets were transferred to 10-cm pots containing quartz sand for acclimatization. Acclimatized plants were transferred to the greenhouse.

**Environmental conditions**

In all experiments, cultures were kept at 25°C. For callus induction and further subcultures, Petri dishes were placed in the dark for induction and further subcultures, Petri dishes were placed in a greenhouse. In all experiments, cultures were kept at 25°C. For callus induction and further subcultures, Petri dishes were placed in a greenhouse.

**Data recording**

After 4 weeks, the number of seeds that induced callus formation was scored and corrected according to seed viability, to determine the callus induction percentage (CIP) (the number of seeds producing callus per 100 viable seeds). Germination percentage data were used according to our previous report (Salehi and Khosh-Khui 2005b).

The number of calli that regenerated shoots and number of shoots produced per explant were counted 4 weeks after their transfer to the regeneration media. The criterion for shoot regeneration was the presence of a number of visible differentiated shoots on callus, having at least one extended leaf (Bai and Qu 2000). Callus regeneration percentage (CRP) was calculated by the number of calli from which shoots were regenerated, divided by the total number of calli transferred to the regeneration medium multiplied by 100. The overall regeneration rate (ORR) was calculated as callus induction percentage × callus regeneration percentage (Bai and Qu 2001). At first, for calculating CRP and ORR only one callus production medium, MS medium supplemented with 60 µM 2,4-D, was used for all genera. Then, the best callus production and shoot regeneration media for each genus were used for calculating CRP and ORR.

**Statistical analysis**

Four replications were used in each treatment with 100 explants in each. All the experiments were repeated at least three times. A CRD with factorial arrangements was used for all the experiments. The interactions between four turfgrass genera and plant growth regulators (BA or 2,4-D) were statistically analyzed. Analysis of variance was carried out using MSTAT-C software (Freed and Eisensmith 1989). Arcsine transformation was carried out on both callus induction and regeneration percentages and overall plant regeneration before analysis. Means were separated using Tukey’s test at the 1% level (P<0.01).

**RESULTS**

**Experiment 1: Effects of seed germination treatments on callus induction in turfgrass genera**

Effects of turfgrass genera or acid, light and dark treatments were studied in this experiment.

**Turfgrass genera**

*Festuca* had the lowest CIP and the results for others were somewhat similar (Table 3). Days to visible callus induction were lowest in *Cynodon* and were similar in other genera (Table 4).

**Acid, light and dark treatments**

Control treatments (without acid) resulted in least CIP and had highest days to visible callus, in both light/dark and dark treatments (Tables 3 and 4).

In general, dark treatment induced more seeds to produce callus than the light/dark treatment. *Cynodon* had greater callus production in light/dark, but with no significant difference with dark treatment (Table 3). However, the quality of *Cynodon* callus in light/dark was better than dark treatment (data not shown). Nevertheless, period to visible callus induction in *Cynodon* was very short in light/dark compared to dark condition (Table 4).

Using acid treatment, resulted close to 100% callus induction percentage (Table 3) and lowest times required to callus induction (Table 4) in all the turfgrass genera.

**Experiment 2: Comparison between callus induction and plant regeneration of turfgrass genera**

The results of this experiment are reported in three separate parts.

**Turfgrass genera**

Among the studied turfgrass genera, *Poa* had the highest CIP regardless of 2,4-D concentration, followed by *Cynodon*, *Lolium*, and *Festuca* (Table 5).

*Cynodon* and *Lolium* had the highest CRP and ORR (Table 6). The highest shoot per explant was produced in *Cynodon*, followed by *Festuca*, *Lolium*, and *Poa* (Tables 7 and 8). The overall mean of shoot production for all the genera was 1.8 in the medium with 7.5 µM BA (Table 7). When, the best callus induction and regeneration media were used for each genus, the CRP and ORR reached nearly 100% in all genera, except *Festuca* (Table 8).
Table 5 Comparison between CIP\(^\text{†}\) of different turfgrass genera cultured on MS medium supplemented with various concentrations of 2,4-D.

<table>
<thead>
<tr>
<th>Turfgrass genera</th>
<th>Mean 2, 4-D ((\mu M))</th>
<th>0</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>150</th>
<th>200</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cynodon(^a)</td>
<td>0 m(^b)</td>
<td>94 bc</td>
<td>100 a</td>
<td>100 a</td>
<td>70 fgh</td>
<td>45 ij</td>
<td>18 k</td>
<td>4 l</td>
<td>54 B</td>
</tr>
<tr>
<td>Festuca</td>
<td>0 m</td>
<td>0 m</td>
<td>0 m</td>
<td>16 k</td>
<td>66 gh</td>
<td>66 gh</td>
<td>78 efg</td>
<td>63 gh</td>
<td>36 D</td>
</tr>
<tr>
<td>Lolium</td>
<td>0 m</td>
<td>0 m</td>
<td>0 m</td>
<td>19 k</td>
<td>78 efg</td>
<td>98 ab</td>
<td>93 bc</td>
<td>93 bc</td>
<td>48 C</td>
</tr>
<tr>
<td>Poa</td>
<td>0 m</td>
<td>90 cde</td>
<td>100 a</td>
<td>93 bcd</td>
<td>84 def</td>
<td>66 gh</td>
<td>60 hi</td>
<td>40 j</td>
<td>66 A</td>
</tr>
<tr>
<td>Mean</td>
<td>0 E</td>
<td>46 D</td>
<td>50 C</td>
<td>57 B</td>
<td>74 A</td>
<td>69 A</td>
<td>62 B</td>
<td>50 C</td>
<td></td>
</tr>
</tbody>
</table>

\(^\text{†}\) CIP\(^\text{†}\)= Callus induction percentage.

Table 6 Comparison between CRP\(^\dagger\) and ORR\(^\ddagger\) of different turfgrass genera cultured on MS medium supplemented with various concentrations of BAP. All the calli were taken from MS medium containing 60 \(\mu M\) 2,4-D.

<table>
<thead>
<tr>
<th>Turfgrass genera</th>
<th>Mean shoots/explant</th>
<th>BAP ((\mu M))</th>
<th>0.0</th>
<th>5.0</th>
<th>7.5</th>
<th>10.0</th>
<th>12.5</th>
<th>15.0</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cynodon(^a)</td>
<td>1.0 f(^c)</td>
<td>1.3 ef</td>
<td>2.9 a</td>
<td>2.1 b</td>
<td>1.4 def</td>
<td>1.4 def</td>
<td>1.4 def</td>
<td>1.7 A</td>
<td></td>
</tr>
<tr>
<td>Festuca</td>
<td>1.0 f(^c)</td>
<td>1.0 f</td>
<td>1.4 def</td>
<td>1.4 def</td>
<td>2.0 b</td>
<td>1.5 cde</td>
<td>1.4 B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lolium</td>
<td>1.3 ef (^c)</td>
<td>1.4 def</td>
<td>1.9 bc</td>
<td>1.3 ef</td>
<td>1.1 ef</td>
<td>1.0 f</td>
<td>1.3 BC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poa</td>
<td>1.0 f(^c)</td>
<td>1.1 ef</td>
<td>1.0 f</td>
<td>1.2 ef</td>
<td>1.8 bcd</td>
<td>1.2 ef</td>
<td>1.2 C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.1 D</td>
<td>1.2 CD</td>
<td>1.8 A</td>
<td>1.5 B</td>
<td>1.6 B</td>
<td>1.3 C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^\dagger\) CRP\(^\dagger\)= Callus regeneration percentage.

\(^\ddagger\) ORR\(^\ddagger\)= Overall regeneration rate.

Table 7 Mean shoot production per explant in different turfgrass genera cultured on media supplemented with various concentrations of BAP. All the calli were taken from MS medium containing 60 \(\mu M\) 2,4-D.

<table>
<thead>
<tr>
<th>Turfgrass genera</th>
<th>Mean shoots/explant</th>
<th>BAP ((\mu M))</th>
<th>0.0</th>
<th>5.0</th>
<th>7.5</th>
<th>10.0</th>
<th>12.5</th>
<th>15.0</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cynodon(^a)</td>
<td>7.3</td>
<td>100 a(^a)</td>
<td>100 a</td>
<td>3.2 a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Festuca</td>
<td>12.5</td>
<td>52 b</td>
<td>41 b</td>
<td>2.5 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lolium</td>
<td>7.5</td>
<td>100 a</td>
<td>98 a</td>
<td>2.2 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poa</td>
<td>12.5</td>
<td>100 a</td>
<td>100 a</td>
<td>2.0 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^\text{†}\) CIP\(^\text{†}\)= Callus regeneration percentage.

\(^\ddagger\) ORR\(^\ddagger\)= Overall regeneration rate.

Table 8 Comparison between CRP\(^\dagger\), ORR\(^\ddagger\) and mean shoot production per explant of different turfgrass genera cultured on the best media for each genus based on data presented in Table 6.

<table>
<thead>
<tr>
<th>Turfgrass genera</th>
<th>Callus induction (2,4-D, (\mu M))</th>
<th>Plant regeneration (BAP, (\mu M))</th>
<th>CRP</th>
<th>ORR</th>
<th>Mean shoot/explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cynodon(^a)</td>
<td>40</td>
<td>7.3</td>
<td>100 a(^a)</td>
<td>100 a</td>
<td>3.2 a</td>
</tr>
<tr>
<td>Festuca</td>
<td>200</td>
<td>12.5</td>
<td>52 b</td>
<td>41 b</td>
<td>2.5 b</td>
</tr>
<tr>
<td>Lolium</td>
<td>150</td>
<td>7.5</td>
<td>100 a</td>
<td>98 a</td>
<td>2.2 b</td>
</tr>
<tr>
<td>Poa</td>
<td>200</td>
<td>12.5</td>
<td>100 a</td>
<td>100 a</td>
<td>2.0 b</td>
</tr>
</tbody>
</table>

\(^\ddagger\) CIP\(^\ddagger\)= Callus regeneration percentage.

\(^\ddagger\) ORR\(^\ddagger\)= Overall regeneration rate.

DISCUSSION

Experiment 1: Effects of seed germination treatments on callus induction in turfgrass genera

Effect of turfgrass genera and acid, light, and dark treatments are discussed in separate parts.

Turfgrass genera

Differences among CIP and days to visible callus induction of the genera used may be due to genotypic variability.

Acid, light, and dark treatments

Control treatments resulted in the least CIP had the highest days to visible callus, in both light/dark and dark treatments. This is in accordance with the seed germination studies in these turfgrass genera that acid treatment enhanced seed germination percentage and mean daily germination (Salehi and Khosh-Khui 2005b). Commercial turfgrass seeds have lemma and palea (Turgeon 2002). Dehusking the seeds by acid treatment caused more direct contact between seeds and culture medium, which could result in better callus induction.

In general, dark treatment induced more seeds to produce callus than light/dark treatment. This is in agreement with Krans et al. (1982) for creeping bentgrass and Bai (2001) for tall fescue. Krans et al. (1982) reported that light...
incubated cultures required more time and effort to excise callus from callorypseus compared to dark treatment. In this study, *Cynodon* had greater callus production in light/dark, but with no significant difference from dark treatment. However, the quality of *Cynodon* callus in light/dark was better than dark treatment. Nevertheless, period to visible callus induction in *Cynodon* was very short in light/dark compared to dark condition. These differences may be due to genotype and/or media variability.

Similar to these results, Conger and Carabia (1978) and Lowe and Conger (1979) reported near to 100% callus induction percentages in acid treated callorypseus of orchardgrass and tall fescue. Acid treated callorypseus of common Kentucky bluegrass also produced 58% to 100% in vitro seed derived calli (McDonnell and Conger 1984).

Acid treatment, especially if seeds are afterward, incubated in dark, may be a useful procedure for in vitro seed culture of *Festuca*, *Lolium* and *Poa*. Light/dark conditions may be suitable for *Cynodon*.

**Experiment 2: Comparison between callus induction and plant regeneration of turfgrass genera**

The results obtained in this experiment are discussed in three separate parts.

**Turfgrass genera**

Regardless of 2,4-D concentration used, *Poa* had the highest CIP. Van der Valk et al. (1995), used mature *Poa* ‘Baron’ and ‘Julia’ seeds, only produced up to 39.3 and 27.6% regenerable callus, respectively. While in the present study, *Poa* produced 100% regenerable callus. Low concentrations of BA in combination with 2,4-D produced the highest regeneration rate (van der Valk et al. 1995), which resulted in browning of callus compared to BA-free media. Therefore, using higher concentrations of 2,4-D not only will probably result in higher CIP, CRP, and ORR, but could also prevent callus browning in *Poa*. Moreover, genotypes differed in the amounts of endogenous hormones and this could result in different observations.

For comparing the genera according to regeneration percentage, rate, or number of shoots produced per explant, only callus produced on MS medium containing 60 μM 2,4-D were used. It made the comparisons easier, because this concentration produced the highest CIP averaged over the genera. Also, residual activity of 2,4-D may be retained in callus tissue after transfer to regeneration media (Zaghmout and Torello 1988), which can negatively affect the callus regeneration ability. Only one callus induction medium was used in the regeneration studies.

The low response of *Festuca* to tissue culture conditions may be due to lower germination percentage of this genus in this study in comparison to other genera.

**Regeneration rate**

The regeneration rate in *Festuca* was lower than other genera in all BA concentrations. As previously discussed, the *Festuca* seed used in this study had a lower germination percentage than other genera. Furthermore, it seems that *Festuca*, like some *Cynodon* (Chaudhury and Qu 2000; Li and Qu 2004) and *Poa* (van der Valk et al. 1995) cultivars, may need low concentrations of BA in the callus induction medium. Further experiments are required for better understanding of the effect of BA on the regeneration rate of *Festuca* callus.

Auxins and cytokinins have adverse effects in tissue culture experiments (Hartmann et al. 2001). It may be concluded that when an explant needs high concentrations of auxins in the callus induction medium, may contain high levels of endogenous cytokinin and will require low concentrations of cytokinin in the regeneration medium. This was an accurate assumption for *Lolium*, *Poa* and somewhat for *Cynodon*, but not for *Festuca*, which needs high concentrations of auxin in callus induction media and cytokinin in regeneration media. Perhaps some genera, like *Festuca*, can easily absorb the exogenous growth regulators and use them rather than endogenous hormones. Further experiments are needed to elucidate this point.

In the present investigation a high regeneration rate in *Cynodon*, *Lolium*, and *Poa* in most of the calli through somatic embryogenesis was observed. In addition, most of the explants regenerated shoots on the media used.

For successful transformation, a large number of embryogenic cells are needed, both regenerable and transformation competent (Li and Qu 2004). Altpeter et al. (2000) observed the highest transformation efficiencies with highly embryogenic cultivars of *Lolium* sp. Therefore, if the best media and culture conditions are used, shoots can be produced from all the calli; if only one piece of transgenic cal- lus is produced, it can be regenerated into a transgenic plant. Furthermore, based on the sequence of transformation study, the same steps may be used for callus production or plant rege- neration in multi-generic transformation studies. In the future, it may be needed to coculture the calli of different genotypes (the same as in vitro nurse-culture) to improve their transformation efficiency. Consequently, MS medium supplemented with 60 μM 2,4-D (for callus induction) and 7.5 μM BA (for regeneration) can be used in multigenic transformation studies with the genera used.

**REFERENCES**


Bai Y, Qu R (2001) Factors influencing tissue culture responses of mature seeds and immature embryos in turf-type tall fescue (*Festuca arundinacea Schreb.*). Plant Breeding 120, 239-242.


Cho MJ, Ha CD, Lemaux PG (2000) Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly re-
generative tissues. Plant Cell Reports 19, 1084-1089
Lee L, Koshy-Khu M (2005a) Effects of genotypes and plant growth regulators on callus induction and plant regeneration in four important turfgrass genera (a comparative study). In: Vitro Cellular and Developmental Biology - Plant, 41, 157-161
Stephens LC, Fei SZ, Xiong Y, Hedges CE (2006) Plants regenerated from embryio cultures of an apomictic clone of Kentucky bluegrass (Poa pratensis L. ‘Baron’) are not apomorphic in origin. Euphytica 147, 383-388
van der Maas HM, de Jong ER, Rup S, Hensgens LAM, Krens FA (1994) Somatic transformation and long-term expression of the gus A reporter gene in cultured cells of perennial ryegrass (Lolium perenne L.). Plant Molecular Biology 24, 401-405