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Tissue Culture and Genetic Transformation of Some Turfgrass Genera

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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

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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

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Invited Mini-Review

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, Bermudagrass, 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 monocots, 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). This is especially true in cereal cell culture (Vasil 1988). In monocots, explants that contain immature, meristematic cells develop a callus that is more likely to express totipotency (Bai 2001). In cereal 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 N^6-benzylamino purine (BAP), in combination with auxins has been reported to enhance embryogenic callus formation and regeneration ability (Zhong *et al*. 1991; van der Valk *et al*. 1995; Chaudhury and Qu 2000; Bai 2001; Salehi *et al*. 2005b).

Genotypic effects

Genotypic effect is a significant factor controlling explant response to *in vitro* culture conditions (Chai and Sticklen 1998). This is observed in tall fescue, *Festuca arundinacea* Schreb. (Bai and Qu 2000) and *Cynodon dactylon* (L.) Pers. (Salehi *et al*. 2005b). Altpeter and Xu (2000) observed significant genotypic differences in callus induction and regeneration frequency and the number of regenerated plantlets per explant in seven cultivars of *F. rubra* L.

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 embryo (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), node segments (Wang and Ge 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.

Plant species	Transgenes	Authors
Common bermudagrass [*Cynodon dactylon* (L.) Pers.]	*gus*, *hpt*	Li and Qu 2004
Red fescue (*Festuca rubra* L.)	*hpt*	Spangenberg *et al*. 1995a
	nptII	Spangenberg *et al*. 1998
	nptII	Altpeter and Xu 2000
	gfp, *gus*, *hpt*	Cho *et al*. 2000
Perennial ryegrass (*Lolium perenne* L.)	*hpt*	Hensgens *et al*. 1993
	gus, *hpt*	Van der Maas *et al*. 1994
	gus, *hpt*	Spangenberg *et al*. 1995b
	gus, *hpt*	Dalton *et al*. 1999
	nptII	Alpeter *et al*. 2000
	nptII, *RgMV-CP*	Xu *et al*. 2001
	Lol p1, *Lol p2*	Petrovska *et al*. 2004
	wft1, *wft2*	Hisano *et al*. 2004
	arg E, *hpt*	Chen *et al*. 2005
Kentucky bluegrass (*Poa pratensis* L.)	*gfp*, *gus*, *hpt*	Ha *et al*. 2001
	bar, *gus*, *hpt*	Gao *et al*. 2006

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

Plant species	Transgenes	Authors
Common bermudagrass	*cry1Ac*, *gus*, *hpt*, *nptII*	Salehi *et al*. 2005b
[*Cynodon dactylon* (L.)	*gus*, *hpt*	Li *et al*. 2005
Pers.]	*gus*, *hpt*	Wang and Ge 2005
Red fescue (*Festuca rubra* L.)	-	-
Perennial ryegrass	*bar*, *gus*, *OsNHX1*	Wu *et al*. 2005
(*Lolium perenne* L.)	*gus*, *hpt*	Bajaj *et al*. 2006
	gus, *hpt*	Sato and Takamizo 2006
	gus, *hpt*	Bajaj *et al*. 2006
	gus, *hpt*	Cao *et al*. 2006
Kentucky bluegrass (*Poa pratensis* L.)	-	-

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 sub-family and Festuceae tribe is a native of Europe (Darlington and Wylie 1961; Beard 1973). This is a largely 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, Eragrostoideae 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 prostrate 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 sterilized distilled 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 δ-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/m^2/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 Eisensmith 1989). Arcsine transformation was carried out on percentage data. Means were separated using Tukey's test at the 1% level ($P \leq 0.01$).

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-

agar (Merck, 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 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 *Festuca*, *Lolium*, and *Poa*, or in a 16/8 h light/dark photoperiod with 140 µmol/m^2/s light from cool white fluorescent lamps for *Cynodon* (Salehi and Khosh-Khui 2003). The same light conditions were used for plant regeneration and rooting.

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 ($P \leq 0.01$).

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**).

Table 3 Callus induction percentages in *in vitro* seed culture of different turfgrass genera with or without acid treatments in light/dark or dark conditions.

Turfgrass genera	Treatments				Mean
	Light/dark		Dark		
	With acid	Without acid	With acid	Without acid	
Cynodon	100 a	60 cde	95 a	49 e	76 B
Festuca	38 f	8 g	92 a	57 de	48 C
Lolium	96 a	71 bc	100 a	72 b	85 A
Poa	93 a	61 bcd	100 a	67 bcd	80 AB
Mean	82 B	50 D	97 A	61 C	

† Data followed by the same lower-case letters (capital letters for mean) are not significantly different according to Tukey's test at 1% level.
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Table 4 Days to visible callus induction in *in vitro* seed culture of different turfgrass genera with or without acid treatments in light/dark or dark conditions.

Turfgrass genera	Treatments				Mean
	Light/dark		Dark		
	With acid	Without acid	With acid	Without acid	
Cynodon	3 f†	10 b	6 e	14 a	8 B
Festuca	11 b	15 a	9 cd	10 bc	11 A
Lolium	11 b	14 a	8 d	10 bc	11 A
Poa	11 b	14 a	8 d	10 bc	11 A
Mean	9 C	13 A	8 D	11 B	

† Data followed by the same small letters (capital letters for mean) are not significantly different according to Tukey's test at 1% level.
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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† of different turfgrass genera cultured on MS medium supplemented with various concentrations of 2,4-D.

Turfgrass genera	CIP								Mean
	2, 4-D (µM)								
	0	30	40	50	60	150	200	250	
Cynodon§	0 m††	94 bc	100 a	100 a	70 fgh	45 ij	18 k	4 l	54 B
Festuca	0 m	0 m	0 m	16 k	66 gh	66 gh	78 efg	63 gh	36 D
Lolium	0 m	0 m	0 m	19 k	78 efg	98 ab	93 bc	93 bc	48 C
Poa	0 m	90 cde	100 a	93 bcd	84 def	66 gh	60 hi	40 j	66 A
Mean	0 E	46 D	50 C	57 B	74 A	69 A	62 B	50 C	

† CIP= Callus induction percentage.
§ Germination percentages were: 100, 85, 98 and 100% for *Cynodon*, *Festuca*, *Lolium and Poa*, respectively.
†† Data followed by the same letters (small letters for columns and rows and capital letters for means) are not significantly different according to Tukey's test at 1% level.
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Table 6 Comparison between CRP† and ORR§ of different turfgrass genera cultured on MS media supplemented with various concentrations of BAP. All the calli were taken from MS medium containing 60 µM 2,4-D.

Turfgrass genera	BAP (µM)													
	0.0		5.0		7.5		10.0		12.5		15.0		Mean	
	CRP	ORR	CRP	ORR	CRP	ORR	CRP	ORR	CRP	ORR	CRP	ORR	CRP	ORR
Cynodon	19 de††	13 def	43 bcde	30 cdef	100 a	70 ab	76 ab	53 bc	46 bcde	32 cdef	41 bcde	29 cdef	54 A	38 AB
Festuca	10 e	7 f	13 e	8 f	19 de	12 ef	19 de	12 ef	34 cde	22 def	24 de	16 def	20 C	13 C
Lolium	55 bcd	43 cd	70 bc	54 bc	100 a	78 a	48 bcde	37 cde	31 de	24 def	23 de	17 def	54 A	42 A
Poa	16 de	14 def	15 e	13 ef	18 de	15 def	30 de	25 cdef	100 a	84 a	34 cde	28 cdef	35 B	30 B
Mean: CRP	25 D		35 CD		59 A		43 BC		53 AB		30 CD			
ORR		19 D		26 CD		44 A		32 BC		41 AB		23 CD		

† CRP= Callus regeneration percentage.
§ ORR= Overall regeneration rate.
†† Data followed by the same letters (small letters for columns and rows and capital letters for means) are not significantly different according to Tukey's test at 1% level.
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Table 7 Mean shoot production per explant in different turfgrass genera cultured on MS media supplemented with various concentrations of BAP. All the calli were taken from MS medium containing 60 µM 2,4-D.

Turfgrass genera	Mean shoots/explant						
	BAP (µM)						
	0.0	5.0	7.5	10.0	12.5	15.0	Mean
Cynodon	1.0 f†	1.3 ef	2.9 a	2.1 b	1.4 def	1.4 def	1.7 A
Festuca	1.0 f	1.0 f	1.4 def	1.4 def	2.0 b	1.5 cde	1.4 B
Lolium	1.3 ef	1.4 def	1.9 bc	1.3 ef	1.1 ef	1.0 f	1.3 BC
Poa	1.0 f	1.1 ef	1.0 f	1.2 ef	1.8 bcd	1.2 ef	1.2 C
Mean	1.1 D	1.2 CD	1.8 A	1.5 B	1.6 B	1.3 C	

† Data followed by the same letters (small letters for columns and rows and capital letters for means) are not significantly different according to Tukey's test at 1% level.
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Table 8 Comparison between CRP†, ORR§ 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**.

Turfgrass genera	Media		CRP	ORR	Mean shoot/explant
	Callus induction (2,4-D, µM)	Plant regeneration (BAP, µM)			
Cynodon	40	7.5	100 a††	100 a	3.2 a
Festuca	200	12.5	52 b	41 b	2.5 b
Lolium	150	7.5	100 a	98 a	2.2 b
Poa	40	12.5	100 a	100 a	2.0 b

† CRP= Callus regeneration percentage.
§ ORR= Overall regeneration rate.
†† In each column, data followed by the same letters are not significantly different according to Tukey's test at 1% level.
Reprinted from **Salehi H, Khosh-Khui M** (2005) 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, with kind permission of the Society for In Vitro Biology, formerly the Tissue Culture Association, ©2005.

Regeneration rate

Based on data presented in **Table 6**, **7**.5 µM BA generally had the best CRP and ORR. The regeneration rate in *Festuca* was lower than other genera for all the BA concentrations (**Tables 6** and **8**).

Most of the explants regenerated shoots on the media used through somatic embryogenesis.

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 caryopses 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 caryopses of orchardgrass and tall fescue. Acid treated caryopses 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), using 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 calli 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 callus is produced, it can be regenerated into a transgenic plant. Furthermore, based on the results of the present study, the same steps may be used for callus production or plant regeneration 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 multigeneric transformation studies with the genera used.

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