EMBRYOGENIC CALLUS INDUCTION AND PLANT REGENERATION MEDIA FOR BENTGRASSES AND ANNUAL BLUEGRASS

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SUMMARY

Embryogenic callus induction and plant regeneration systems have long been established for creeping bentgrass (Agrostis palustris Huds.), but little research has been reported on optimal medium for embryogenic callus induction and plant regeneration in velvet bentgrass (Agrostis canina L.), colonial bentgrass (Agrostis capillaries L.), and annual bluegrass (Poa annua L.). The present study compared 14 callus induction media and eight regeneration media for their efficacies on embryogenic callus induction and plant regeneration in these four species. The embryogenic callus initiation media contained the Murashige and Skoog inorganic salts and vitamins supplemented with 2,4-dichlorophenoxyacetic acid or 3,6-dichloro-anisic acid and 6-benzyladenine. L-Proline or casein hydrolyzate was included in some media to stimulate embryogenic callus formation and plant regeneration. The frequencies of embryogenic callus formation ranged from 0% to 38% and exhibited medium differences within each of the four species. Callus induction media, plant regeneration media, and genotypes affected plant regeneration rates, which varied between 0% and 100%. The embryogenic callus induced on Murashige and Skoog medium supplemented with 500 mg l\(^{-1}\) casein hydrolyzate, 6.63 mg l\(^{-1}\) 3,6-dichloro-anisic acid and 0.5–2.0 mg l\(^{-1}\) (2–9 \(\mu\)M) 6-benzyladenine had much higher regeneration rates than those formed on other callus induction media. Embryogenic callus of annual bluegrass had higher regeneration rates than those of bentgrass species. MSA2D, a media containing 2 mg l\(^{-1}\) (8 \(\mu\)M) 2,4-dichlorophenoxyacetic acid, 100 mg l\(^{-1}\) myo-inositol, and 150 mg l\(^{-1}\) asparagine, was effective in promoting embryogenic callus formation in creeping bentgrass but not in colonial and velvet bentgrasses and annual bluegrass.

Key words: Agrostis palustris; Agrostis canina; Agrostis capillaries; Poa annua; tissue culture media; frequency of embryogenic callus formation.

INTRODUCTION

To improve creeping bentgrass (Agrostis palustris Huds.), embryogenic callus has been used as target tissue for biotic and Agrobacterium-mediated transformation (Zhong et al., 1993; Lee, 1996; Lee et al., 1996; Warkentin et al., 1997; Liu et al., 1998; B. Zilinskas, personal communication). The effectiveness of these transformation approaches largely depends on the quality and quantity of embryogenic callus, among other factors. Embryogenic callus formation is influenced by many factors, among which culture medium composition is particularly important. MS medium (Murashige and Skoog, 1962) is frequently used for turfgrass tissue culture and the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) or 3,6-dichloro-anisic acid (dicamba) plus 6-benzyladenine (BA) are often added to MS medium, depending on the grass species (Lowe and Conger, 1979; Torello et al., 1984; Dalton, 1983; Jackson and Dale, 1983; Zhong et al., 1991; Zaghmout and Torello, 1992; Wang et al., 1993; Hartman et al., 1994; Lee et al., 1996; Warkentin et al., 1997). To stimulate embryogenic callus formation in redtop (Agrostis alba L.) and creeping bentgrasses, some researchers used l-proline and casein hydrolyzate in callus initiation media (Shetty and Asano, 1991; Zhong et al., 1991). myo-Inositol and asparagines have been used for embryogenic callus initiation and plant regeneration in producing transgenic creeping bentgrass (Hartman et al., 1994; Lee et al., 1996). MS medium supplemented with 2,4-D but not amino acids has also been used for callus induction in creeping bentgrass (Krans et al., 1982; Blanche et al., 1986). It appears that the optimum medium varies substantially with grass species.

Velvet bentgrass (Agrostis canina L.) and colonial bentgrass (Agrostis capillaries L.) will likely become important turfgrass species for genetic enhancement through gene transfer methods because they have fine texture and low management requirements, but are susceptible to various stresses, such as disease, heat, and drought. The optimum callus induction and plant regeneration conditions for these two species have not been reported. Annual bluegrass (Poa annua L.) is often considered a weed in golf course management, but this species exhibits high genetic variability, rapid germination, short life cycle, soil compaction tolerance, low
temperature tolerance, and quick death in warm weather (McCarty, 1999). These characteristics may be useful for turfgrass improvement via genetic transformation. There has been no report on optimum media for callus induction and plant regeneration for this species.

The study reported here was to compare different media for their efficiencies on embryogenic callus formation and plant regeneration in velvet bentgrass, colonial bentgrass, and annual bluegrass. The objective was to find species/cultivar-specific concentrations and combinations of 2,4-D, dicamba and BA, l-proline, casein hydrolyzate, myo-inositol, and asparagines. The results of this study will be useful for genetic transformation of these turfgrasses. Creeping bentgrass was included in this study as reference species because the optimum conditions for its callus production and plant regeneration have been well established (Zhong et al., 1991).

**Materials and Methods**

**Plant materials and culture media.** Mature seeds (caryopses) of annual bluegrass, common type; velvet bentgrass, cvs. SR7200 colonial bentgrass, cv. Exeter, and creeping bentgrass, cvs. Penn A-4 and Providence, were surface-sterilized for 20 min with a solution containing 20% (v/v) commercial Clorox bleach (active ingredient 5.25% NaClO) and 0.5% Tween 20 (polyoxyethylene-sorbitan monolaurate). The sterilized seeds were rinsed three times with sterilized distilled water before being transferred to autoclaved semisolidified callus induction media. The media contained MS basal salts, vitamins, and 3% (w/v) sucrose, supplemented with specified concentrations of various plant hormones and amino acids (Table 1). All media were adjusted to pH 5.6–5.8 prior to the addition of 0.2% phytagel (Sigma Chemical Co., St. Louis, MO) and autoclaved at 121°C for 20 min.

The cultures were incubated for 6–7 wk at room temperature (24 ± 2°C) in darkness. Only friable, compact, organized, white to pale yellow embryogenic callus (Vasil, 1987) was subcultured or transferred to Petri dishes (15 x 100 mm) containing 25 ml of regeneration medium for plant regeneration. These cultures for plant regeneration were incubated for 1 wk in darkness and then for 4 wk under fluorescent light (60 µE m⁻² s⁻¹, 16/8 h day/night). Regenerated plants were transferred to culture flats containing 1:1 mixture of sphagnum peat moss and vermiculite, and maintained in a greenhouse (sunlight, 17–35°C).

**Data collection and statistical analysis.** Each cultivar was seeded into five Petri dishes (15 x 100 mm) per medium (five replicates), and each dish contained 20 seeds. Number of viable seeds, number of callus clumps, and number of visible embryogenic calluses were recorded for each Petri dish 6–7 wk after initial culture. The frequency of callus or embryogenic callus formation in a Petri dish was expressed as the percentage of callus clumps or visible embryogenic calluses per total number of viable seeds in that Petri dish, respectively. The frequency of plant regeneration was expressed as the percentage of callus clumps that produced plants per total number of embryogenic callus in each Petri dish.

Analysis of variance was performed using the SAS for Windows program (SAS Institute, Cary, NC). Significant means were separated using Duncan’s multiple range test. Minitab was used to analyze significance of correlation.

**Results and Discussion**

In previous studies, the frequency of callus formation was expressed as the percentage of the number of calluses produced per total number of seeds sown in a given Petri dish (Shetty and Asano, 1991; Zhong et al., 1991). The callus formation rate defined as above may be skewed by seed viability because only viable seeds may produce calluses on a suitable medium. This expression may be suitable when comparing callus formation rates of a single species provided that the seeds used have the same viability. In the present study, we chose to express the frequency of callus formation as the percentage of callus produced per total number of viable seeds in each Petri dish. A viable seed was the one that germinated or produced callus on callus induction media. This method of calculation excluded the bias resulting from seed viability differences among different species and cultivars. Frequencies of callus formation based on the total number of viable seeds (Table 2) were generally higher than frequencies based on the total number of sown seeds because the viability of turfgrass seeds is usually lower than 100%.

Similarly, the frequency of embryogenic callus formation has been previously expressed as the percentage of embryogenic calluses produced per total number of seeds sown (Wang et al., 1993) or calluses produced (Ahn et al., 1987; Shetty and Asano, 1991; Zhong et al., 1991) in each Petri dish. Because of the aforementioned potential complication from seed viability differences, we chose not to use total number of seeds sown in a dish as the basis for calculating the frequency of embryogenic callus formation. Furthermore, we did not use total number of calluses as the basis, because the frequency of embryogenic callus formation per total number of calluses can be distorted by the frequency of callus formation. For example, data of Ahn et al. (1987) showed that 40 explants (immature inflorescences) of ‘Tifway’ bermudagrass produced four calluses and subsequently two embryogenic calluses, while 30 explants of common bermudagrass produced 26 calluses and subsequently four embryogenic calluses. Based on the number of calluses, the frequency of embryogenic callus formation was 50% for ‘Tifway’ bermudagrass and 15% for common bermudagrass, as reported originally. However, these percentages alone could not represent the real difference in the capacities of embryogenic callus formation between ‘Tifway’ and common bermudagrass since ‘Tifway’ produced only two embryogenic calluses out of 40 explants, at a rate of 5% based on number of explants, while common bermudagrass produced four embryogenic calluses out of 30 explants, at a rate of 13%. As the explants in our experiments were seeds and the immediate objective was to obtain embryogenic calluses from seeds, we decided to express the frequency of embryogenic callus formation on the basis of total viable seeds in each Petri dish.

**Influence of the media on callus formation.** The frequency of callus formation varied significantly among cultivars (P < 0.01) and among media (P < 0.01). Interactions between cultivar and medium were also significant (P < 0.01). Duncan’s multiple range tests showed that the three MSCDB media containing dicamba and BA in addition to casein hydrolyzate had the lowest frequency of callus induction in the bentgrass cultivars, ranging from 13% to 53% (Table 2). The MSCDB medium containing 2 mg l⁻¹ BA (MSCDB2) resulted in a lower callus induction rate than the other two MSCDB media with lower concentrations of BA (MSCDB05 and MSCDB1), indicating an inhibitory effect of BA on callus induction in bentgrasses. Other callus induction media exhibited the same effect on callus induction in the bentgrass cultivars, except MSNA2D in ‘Penn A-4’. Annual bluegrass had lower frequencies of callus formation than the bentgrasses on all media except MSDP1 and MSDP2. The MSDP media produced higher frequencies of callus induction for annual bluegrass than other media, which suggested that l-proline at a concentration of 1 or 2 g l⁻¹ promoted callus induction in annual bluegrass.

These callus induction media produced several morphological types of callus. Friable embryogenic callus (Fig. 1A, B) was similar to ‘Type II’ callus of maize (Zea mays) (Armstrong and Green, 1985;