EFFECT OF PLANT GROWTH REGULATORS ON CALLUS INDUCTION AND SHOOT REGENERATION OF THE KENTUCKY BLUEGRASS (*Poa pratensis* L.)

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Abstract. Kentucky bluegrass is one of the most important species of cold season grass which is used in mixed sports turfgrass. This study was conducted to determine the effect of plant growth regulators on callus induction and plant regeneration from hypocotyl explants in Kentucky bluegrass. For this purpose, the MS media containing two auxins (2,4-D and NAA) at different concentrations of 0.5, 1, 2, 3, 4 and 5 mg/L were used alone or in combination with and without the 0.1 mg/ L Benzyladenine (BA). The results showed that the highest callus induction rate was obtained at concentrations of 2 mg/L 2,4-D in combination with 1.0 mg/L BA or 1 mg/L NAA alone. The highest plant regeneration rate was obtained at a concentration of 0.5 mg/L 2,4-D. The protocol we introduced can be used for rapid and scalable micropropagation of this turfgrass species and may also serve as a good platform for for protocol developments of other *recalcitran*t turfgrasses.

Keywords: auxin; cytokinins; hypocotyl; turfgrass.

INTRODUCTION

To receive successful transgenic technologies on forage grasses, an effective *in vitro* culture system is essential. Since, use of biotechnological tools including *in vitro* culture or genetic transformation may be considered for the improvement of quality and enhancement of resistance to different abiotic or biotic stresses of forage [17] .Tissue culture is used for various purposes such as rapid propagation and the creation of somaclonal varieties in breeding projects. *In vitro* culture techniques play a basic role in turfgrass breeding [4]. Although cell and tissue culture methods have been used in many plant species, less attention has been paid to turfgrass in this field. In general, monocotyledons have been found to be less studied in *in vitro* culture studies than dicotyledons [36]. Many factors, such as the type of explants, the culture medium, and plant growth regulators, amino acids etc., affect the response of *in vitro* turfgrass, especially the callus formation and its regeneration [4]. The common problem for *in vitro* culture of turfgrasses and other herbaceous species is the natural disability for secondary growth by cambium and cambio-like tissues in mature and differentiated explants. For this reason, these plants such as turfgrass are classified as *recalcitrant* plants for *in vitro* culture conditions [36].

Among various plant growth regulators, 2,4-Dichlorophenoxyacetic acid (2,4-D) is known as an auxin which has been used in many plant species for the induction and growth of callus [27]. The effect of 2,4-D on callus induction can be attributed to its key characteristic of stimulating cell division from plant tissues and severe inhibition of organogenesis. Plant growth regulators are synthetic molecules that are used in plants and applied almost at low concentrations to act as signaling compounds for plant growth and development [29]. Zong et al. [36] used three Kentucky bluegrass cultivars, in MS medium supplemented with 2,4-D (1, 2, 3 and 4 mg/L) and benzyl aminopurine (BA) (0.1, 0.2 and 0.3 mg/L) for induction of callus. The authors observed the best induction of callus in a culture medium containing 2 mg/L 2,4-D alone and 3 mg/L 2,4-D in combination with 0.1 mg/L BA. The same culture medium with 0.1, 0.2, 0.4, 0.8 and 1 mg/L, 2,4-D was used for the induction of callus from nodes in three species of Kentucky grass, Reygrass and Cynodon, and the best concentration 2,4-D was 1 mg/L 2,4-D [31]. The certain auxin levels in combination with low levels of BA improve the rate of callus induction in the eplants [11]. The genotype that need high concentrations of auxin in callus induction medium and requires low concentrations of cytokinin in callus and regeneration media, either might have high levels of internal cytokinin [28].

Therefore, the combination of optimal concentrations of different auxins and cytokinins can lead to better results than the independent effect of these hormones [28]. Ke and Lee [14] investigated the plant regeneration of Kentucky bluegrass on a culture medium containing 0.1 mg/L BA and 0.2 mg/L Picloram or 0.1 mg/L 3-Indole acetic acid (IAA). Lee et al. [17] to optimize appropriate plant growth regulators and their optimum concentration and combinations for seed-derived callus induction of mature seeds of *Dahurian wildrye* grass (*Elymus dahuricus* L.), different kinds of auxins [2,4-D, Dicamba and α-naphthaleneacetic acid (NAA)] alone or in combination with BA were used. The result consisted of 3.0 mg/L 2,4-D or Dicamba in MS showed the highest percentage of callus induction. Highest (51%) regeneration was obtained from N6 medium containing 1.0 mg/L 2,4-D in combination with 3.0 mg/L BA combination for plant regeneration in the *Dahurian wildrye* grass [18].

Kentucky bluegrass (*Poa pratensis* L.) is a typical cool-season perennial grass with apomictic reproduction [2]. The proliferation of this type of turfgrass, like other grasses species, is always subject to limitations due to its *recalcitrance* in *in vitro* conditions. Although different studies have been done to induce *in vitro* cultures of this species, there are few reports of callus induction from different cultivars of this *recalcitrant* turfgrass [35]. The use of

biotechnology in the breeding of various Kentucky varieties was effective so far and proposed for other species [13]. Extensive research on biotechnology and tissue culture led to the investigation of several methods for regeneration of turfgrass cultivars and genotypes from mature seeds [10], suspensions cultures [26] and protoplast cultures [25].

In this study, callus induction and plant regeneration of Kentucky bluegrass was investigated using hypocotyl segments as explants on culture medium with different concentrations and combinations of auxins and cytokinins in order to develop a long-term stable tissue culture system for appropriate for its biotechnological progress.

MATERIALS AND METHODS

Seeds of Kentucky bluegrass (*Poa pratensis* "Barimpala") were obtained from a commercial company. To disinfect, the seeds were immersed in 70% alcohol for two minutes, followed by immersion in 30% (v/v) Clorox ^{TM} solution (active chlorin 5%) for 15 minutes and then were washed three times with sterile distilled water. The seeds were cultured on germination media with 7 g/L of agar, free of plant growth regulators (PGRs) and sucrose for germination. The explants used were hypocotyl segments (5 mm) from germinated seeds (after 14 days).

In this study were used two type of auxins (2,4-D and NAA) at different concentrations (0.5, 1, 2, 3, 4 and 5 mg/L), alone and in combination with or withouth BA (0.1 mg/L). Explants were cultured on a MS medium containing 30 g/L sucrose and 7 g/L of agar with different concentrations of plant growth regulators for callus induction or regeneration. The cultures were placed in a growth chamber under conditions of absolute darkness and temperature of 24±1 °C for callus induction and plant regeneration. At the end of 4th week after inoculation, the amount of callogenesis and shoot regeneration were recorded. The observed parameters for the callus and shoot induction were callus induction percentage (%), diameter (cm) of callus, callus apparent growth and shoot regeneration percentage (%). The percentage of callus induction and plant regeneration for each treatment was calculated according to Alonso-Herradaa et al. [1] method:

$$CI=(n/N)x100$$

where: N - the total number of explant, n - the number of explant that are induced of the callus, and CI - the percentage of callus induction or percentage of regeneration, respectively.

Due to the asymmetric growth of callus, the largest and smallest diameter of callus was measured as a index of the growth of callus. Also, induced calli were needed to advance the regeneration of the shoot. Therefore, it was not possible for them to perform destructive experiments and measure biomass. So, qualitative measurements were used, such as callus apparent growth. Callus apparent was ranked with the following system: no callogenesis=0, low callogenesis=1, medium callogenesis=2 and high callogenesis=3 in each explant.

In order to investigate the effect of different types and concentrations of plant growth regulators on callus induction from hypocotyl, a completely randomized design (CRD) with factorial arrangements, 5 replications and 10 observations per replication was used for each experiment. Data analysis was performed using SAS software. Duncan's Multiple Range Test (DMRT) was used to compare the mean and to determine the significance of statistical differences in treatments at 5% level.

RESULTS

A decrease in the percentage of callus induction and plant regeneration was observed with increasing concentration of 2,4-D (Table 1). The callus induction frequency varied from 12.5 to 92.5 %.The highest percentage of callus induction was obtained for a combination of 2 mg/L 2,4-D with 0.1 mg/L BA (Fig. 1A), while using 5 mg/L 2,4-D (highest concentration) with no BA led to the lowest percentage of callus induction (Table 1). The callus induction rate increased when 2,4-D concentration increased from 0.5 to 2 mg/L. However, at concentrations higher than 2 mg/L, the percentage of callus induction decreased and the lowest concentration was observed at 5 mg/L 2,4-D. The highest plant regeneration rate from callus was observed at 0.5 mg/L 2,4-D. The use of high concentrations of 2,4-D had a negative effect on the plant regeneration rate of calli, so that no regeneration was observed at concentrations higher than 1 mg/L 2,4-D (Table 1). Characteristics of the diameter and callus induction time of callus were also reduced with increasing 2,4-D concentration. However, the best results in terms of maximum diameter and minimum diameter of callus were for concentrations of 1 and 2 mg/L 2,4-D in combination with BA and the highest callus diameter was obtained at a concentration of 0.5 mg/L 2,4-D in combination with BA. The maximum apparent growth of callus was obtained from the explant cultured on MS medium supplemented with 1 to 4 mg/L 2,4-D concentrations. The results showed that there was a significant variation among calli, depending on 2,4-D and BA applications. The best callus formation was observed at 1-2 mg/L 2,4-D concentrations. Low concentration of BA (0.1 mg/L) in the medium showed slightly higher callus formation than 0 mg/L BA concentration. Green shoots were observed within two to three weeks of culture (Fig. 1B).

According to the results of this study, the best treatment for induction of callus was 2 mg/L 2,4-D in combination with 0.1 mg/L BA and the best concentrations for plant regeneration was at concentration of 0.5 mg/L 2,4-D. There was no significant difference between the concentrations of 1 to 5 mg/L NAA for the diameter and apparent growth of callus (Table 2). Minimum diameter and apparent

Table 1. Effect of 2,4-D and BA on callus properties and plant regeneration in Kentucky bluegrass

2,4-D (mg/L)	BA (mg/L)	Callus induction (%)	Shoot regeneration (%)	Max. diameter of callus (mm)	Min. diameter of callus (mm)	Mean diameter of callus (mm)	Apparent growth of callus
0.5	0	90.00 ^{a}	17.50 ^{a}	2.48 ^{b}	1.84 ^{ab}	2.16 ^{bcd}	2.60 ^{ab}
	0.1	50.00 ^{b}	0.00 ^{b}	2.72 ^{ab}	2.13 ^{ab}	3.63 ^{a}	2.60 ^{ab}
1	0	82.50 ^{a}	5.00 ^{b}	2.69 ^{ab}	1.92 ^{ab}	2.30 ^{bc}	3.00 ^{a}
	0.1	80.00 ^{a}	7.50 ^{b}	3.22 ^{ab}	2.19 ^{a}	2.60 ^{ab}	3.00 ^{a}
2	0	80.00 ^{a}	0.00 ^{b}	3.66 ^{a}	1.76 ^{ab}	2.71 ^{ab}	3.00 ^{a}
	0.1	92.50 ^{a}	0.00 ^{b}	3.54 ^{a}	1.64 ^{ab}	2.69 ^{ab}	3.00 ^{a}
3	0	20.00 ^{c}	0.00 ^{b}	2.96 ^{ab}	1.81 ^{ab}	2.38 ^{bc}	3.00 ^{a}
	0.1	78.13 ^{a}	0.00 ^{b}	2.50 ^{b}	1.52 ^{b}	2.01 ^{bcd}	3.00 ^{a}
4	0	47.50 ^{b}	0.00 ^{b}	2.78 ^{ab}	2.06 ^{ab}	2.42 ^{abc}	3.00 ^{a}
	0.1	15.00 ^{c}	0.00 ^{b}	1.42 ^{c}	0.92 ^{c}	1.17 ^{cd}	1.50 ^{c}
5	0	12.50 ^{c}	0.00 ^{b}	1.15 ^{c}	0.88 ^{c}	1.02 ^{d}	1.50 ^{c}
	0.1	50.00 ^{b}	0.00 ^{b}	2.41 ^{b}	1.36 ^{ab}	1.62 ^{bcd}	2.00 ^{bc}

Means followed by the same letters are not significantly different from each other ($p<0.01$) as determined by Duncan's Multiple Range Test (DMRT)

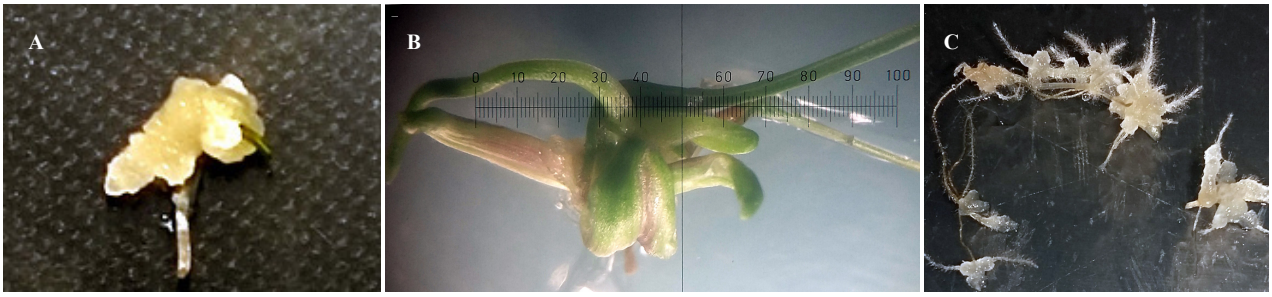

Figure 1. Callus induction and plant regeneration of Kentucky bluegrass: A) callus on a medium with 2,4-D; B) plant regenerated in a culture medium with 0.5 mg/L 2,4-D; C) callus on a medium with 2-3 mg/L NAA.

Table 2. Effect of NAA on callus properties in Kentucky bluegrass

NAA (mg/L)	Max. diameter of callus (mm)	Min. diameter of callus (mm)	Mean diameter of callus (mm)	Apparent growth of callus
0.5	0.99 ^{b}	0.65 ^{b}	0.82 ^{b}	0.60 ^{b}
1	1.89 ^{a}	1.22 ^{a}	1.55 ^{a}	1.20 ^{a}
2	2.19 ^{a}	1.36 ^{a}	1.73 ^{a}	1.50 ^{a}
3	2.01 ^{a}	1.38 ^{a}	1.69 ^{a}	1.50 ^{a}
4	1.92 ^{a}	1.50 ^{a}	1.71 ^{a}	1.62 ^{a}
5	1.93 ^{a}	1.43 ^{a}	1.68 ^{a}	1.25 ^{a}

Means followed by the same letters are not significantly different from each other ($p<0.01$) as determined by Duncan's Multiple Range Test (DMRT).

growth rate of Kentucky bluegrass were 0.5 mg/L NAA.

The comparison of the interaction between NAA and BA on callus induction showed a decrease in the percentages of callus with increasing NAA concentration. The concentrations of 0.5 to 3 mg/L NAA without BA showed a better callus induction percentage with 88.02% on 1 mg/L NAA without BA. There was no significant difference between the concentrations of 2 and 3 mg/L NAA in combination without BA (Figure 2C). The lowest callus induction (2%) was observed on culture medium with 0.5 mg/L NAA in combination with 0.1 mg/L BA.

In all NAA concentrations, the presence of BA did not have a significant effect on callus induction. The interaction of these treatments on other callus formation properties was not significant. The best treatment for inducing callus in Kentucky bluegrass was the culture medium with 1 mg/L NAA and without any cytokinins.

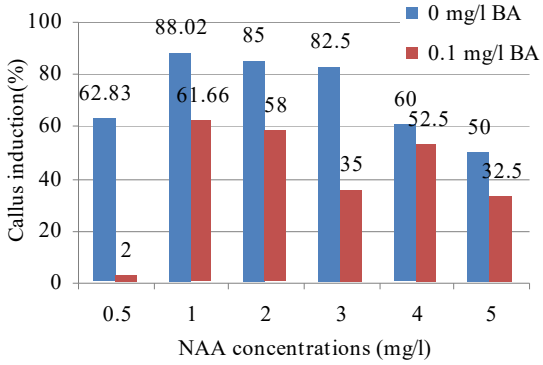

Figure 2. Comparison of means on the effect of NAA and BA on callus induction (%) in Kentucky bluegrass. Means followed by the same letters are not significantly different from each other ($p<0.01$) as determined by Duncan's Multiple Range Test (DMRT).

DISCUSSION

Undifferentiated and unorganized mass of cells formed by the enhancement of parent tissue recognized as callus. Callus mass is a good indication of genetic variability such as somaclonal variation. Auxins are plant growth regulators that stimulate cell division, cell growth expansion, and organization of meristems for callus development in plants tissue culture. Exogenous application of auxin and cytokinin induces callus in various plant species. An intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively [11, 12].

According to the current study on the effect of plant growth regulators on callus induction, callus characteristics and plant regeneration in Kentucky bluegrass, the highest callus induction percentage, the highest diameter and apparent growth of callus was found for the treatment with 2 mg/L 2,4-D with 0.1 mg/L BA and 1 mg/L NAA alone. The highest percentage of plant regeneration was observed at concentration of 0.5 mg/L 2,4-D and decreased with increasing 2,4-D concentration. The other concentrations of NAA did not lead to plant regeneration response in this study.Most of studies on induction of callus in different genus of turfgrass are related to the application of 2,4-D, NAA, IBA, dicamba. 2,4-D alone or in combination with cytokinins is widely used for induction and growth of callus [35] and especially for cereal and turfgrass *in vitro* cultures [3]. The effect of 2,4-D on callus induction in various plants has been reported by many researchers [16]. In a similar studies, 2,4-D was used to induce callus in *Lolium* sp. [33], Orchard grass [16], festuca [20], Centipede grass [23] and Bermuda grass [31].Cui et al., [8] reported that optimal concentrations of 2,4-D for induction and maintenance of callus in turfgrasses were 0.5 to 1 mg/L for Dune reeds and 2 to 3 mg/L for Swamp reeds (Poaceae family). In addition, Lauzer et al. [15] showed that the culture medium supplemented with 1 mg/L 2,4-D caused callus in Reedgrass.

We showed the callus induction, however, varied within different levels of concentrations. Low concentration levels of 2,4-D and NAA was observed to have increased induction rates, whereas higher concentration levels resulted in reduced induction rates. The level of concentration of the respective PGRs was observed to have an important effect on the rate of callus induction, with genotypes revealing different reaction patterns with low concentration levels of 2,4-D resulting in higher induction rates in some turfgarass [8, 15].

Lee et al. [15] indicated that the increasing concentration of 2,4-D in the callus initiation medium resulted in a lower regeneration frequency of callus induction for perennial ryegrass, which was coincident with the results obtained in our study. Lee et al. [19] stated that at 5 mg 2,4-D the rate of callus induction increased, but concentrations higher than 5 mg/L had an adverse effect on callus induction in *Lolium multiflorum*.

Contrary to the results of current experiment, the results of previous experiments showed that the use of high concentrations of auxin would result in an increase in the quality and quantity of callus, but excessive increase would have an adverse effect on calli [5] . This indicates that different types of genuse contain varying levels of endogenous auxin concentrations and callus induction ability is determined by their endogenous hormone variability.

Auxins are used in combination with cytokinins, specifically BA, to stimulate callus induction in *in vitro* conditions. However, the concentration of these plant growth regulators should be defined for each plant species [24]. The ratio of auxin / cytokinin improves callus induction, callus quality, and regeneration ability in legumes and grasses, although this effect may also depend on the levels of cytokinin [3]. It has been reported that the presence of BA in callus induction media inhanced the callus quality and regeneration ability in several legumes and grain species, including barley [7], Bentgrass [34], Kentucky bluegrass [32] and Bermuda grass [6].

Improvement of regeneration rate with low levels of BA in combination with specific auxin in callus induction medium has been reported in some grass species [10, 17, 34]. In this study, the use of BA with NAA resulted in decreased callus induction while using 2,4-D with low BA concentration (0.1 mg/L) has promoted the callus induction. Li et al., [22] also pointed out that for induction of callus from node segments in Zoysia grass, application of 2.4-D alone or in combination with 0.1 mg/L IAA, BA and Kinetin, while for plant regeneration a concentration of 0.2 mg/L BA was effective. Optimal concentration of BAP for callus induction of turfgrass varied widely. For example, for bermudagrass (*Cynodon dactylon*), the optimal concentration of BAP was rather low, only 0.01 mg l-1 (9), while for Kentucky bluegrass (*Poa pratensis* L.) and tall fescue, it was 0.1-0.3 mg l-1 (2, 25) and rather high concentration of BAP (0.5-2 mg l-1) was needed for creeping bentgrass (*Agrostis stolonifera* L.) (29). Dhandapani et al. [9] showed that the highest percentage of callus induction was obtained from the Zoysia grass inflorescences by concentrations of 4.5-4.9 mg/L 2,4-D in combination with 0.44 mg/L of BA. They also used MS medium supplemented with 4.44 to 8.8.8 µM BA, 2.68 µM NAA and 0.28 µM GA3 for induction of callus from Zoysia. Some researchers confirmed the presence of low BA and auxin concentrations in grasses callus induction, including Chaudhury and Qu [6] in Bermudagrass, Bradley [4] in Reygrass and Taghizadeh [30] in Bermudagrass, and these results were confirm with our results on callus induction.

In this case, the maximum callus induction (up to 92%) were obtained from the explant cultured on MS

medium supplemented with 2,4-D (up to 88%) rather. than NAA In plant cells, for auxins to be effective, the PGR has to be protected from oxidative denaturation through molecular conjugation enabling storage and consequential, gradual release for enzymatic action [23]. 2,4-D, therefore, is ideal for callus formation compared to NAA when used singly.

Based on the results of this study, the application of 0.5 mg/L 2,4-D alone showed the highest regeneration rate, which was consistent with the results of previous experiments in relation to improving regeneration in the presence of s Cytokinin was not like BA. Overall, the results of this study showed that the best PGRs composition for inducing callus from the hypocotyl explants was obtained using 2 mg/L of 2,4-D in combination with 0.1 mg/L BA. However, callus induction was observed also on medium with concentrations less than 2 mg/L 2,4-D in the presence or absence of BA.

The combination of cytokinin and auxins is essential for indirect shoot regeneration. The types of organ will be regenerated from undifferentiated callus tissue is determined by the ration of cytokinin to auxin. In general, when callus cultured on media containing high cytokinin to low auxin ratio extreme numbers of shoots and least number of roots were generated, while few shoots and many roots were observed when callus tissue were cultured on media containing low cytokinin to high auxin ratio [32].

The following aspects contributed to the achievement of the system established in the present study for tissue culture of Kentucky bluegrass; it is also predictable to be implemented in some other grass species: First, the low level of auxin (2,4-D) in combination with a low level of cytokinin (BA) will be useful for the callus production. Second, we established tissue culture systems that will enable efficient achievement of regenerated plants from Kentucky bluegrass mature seeds. Third, the system established in the present study for tissue culture of Kentucky bluegrass can get enough callus plant regeneration efficiency to perform transgenic operation. Therefore, in this study, the concentration of 2 mg/L 2,4-D in combination with 0.1 mg/L BA or 1 mg/L NAA for induction of callus is recommended. The protocol we introduced here can be used for rapid and scaleable micropropagation of this turfgrass species and may also serve as a good platform for protocol development of other *recalcitrant* turfgrass.

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