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Culture media and growth regulators influence callus induction and plant regeneration of mature embryos of orchardgrass (*Dactylis glomerata* L.)

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Abstract: To establish an efficient in vitro regeneration protocol for orchardgrass (*Dactylis glomerata* L.), type of medium, several plant growth regulators and their various concentrations were evaluated in a factorial experiment for callus induction and plant regeneration. Endosperm supported mature embryo were used as explants and optimum response in callus induction was observed on Chu medium with 12 mg/L dicamba (3.6-dichloro-2-methoxybenzoic acid). The highest responding embryogenic callus (15%) was found with Murashige and Skoog medium and 8 mg/L 2.4-D (2,4-dichlorophenoxy acetic acid). The highest regeneration efficiency was achieved on MS medium (17 plantlets) with 8 mg/L 2,4-D. Endosperm-supported mature embryos of orchardgrass can be successfully used as explants. Therefore, this protocol provides a basis for future studies on genetic improvement and in vitro mutation studies.

Key words: Culture media, dicamba, picloram, plant regeneration, 2.4-D

1. Introduction

Orchardgrass is a perennial cool-season forage species that is grown in temperate regions of the world to produce high quality hay. Breeding and releasing new orchardgrass varieties with higher forage quality and yield is fundamental for animal feeding. It is highly important that local or domestic varieties of orchardgrass be developed, as there are significant adaptation problems such as low or unsatisfactory yield of exotic lines. However, orchardgrass is genetically self-incompatible, and that makes breeding of new varieties difficult and time consuming (Atis et al., 2013). Biotechnological methods are used efficiently along with classical breeding for many crops. These methods shorten the breeding time and elevate the breeding efficiency. Plant tissue culture methods are commonly used to create new variations that can be used for selection or to transfer the new gene into plant genome (Avcıoğlu et al., 2009).

Plant regeneration efficiency depends on genotype, explant type, growth conditions of donor plant and type of culture medium (Aydin et al., 2011). Various explants such as immature embryo (Arzani and Mirodjagh, 1999), anther (Christensen et al., 1997), leaf (Somleva et al., 1995), (Kuklin and Conger, 1995) and mature seed (Lee et al., 2000; Bae et al., 2002; Lee et al., 2002) are used to initiate the callus formation. The fact that the use of immature embryo as the explant resource is more complicated, and requires more time and expense, limits the efficiency of the system. For this reason, the use of the mature embryo as an alternative explant resource is considered more advantageous. The supply and storage of seeds as the source of the mature embryos provides the opportunity of working throughout the year (Birsin and Ozgen, 2004) Although both nonendosperm-supported mature and endosperm-supported mature were used for plant regeneration via somatic embryogenesis, endosperm-supported mature were determined superior in terms of plant regeneration (Aydin et al., 2016; Aydin et al., 2011).

Plant growth regulators are one of the other important factors affecting in vitro plant regeneration. Auxins stimulate the cell division and development and affecting callus and somatic embryo formation (Weijers and Jürgens, 2005). The auxin types such as 2,4-D and NAA (1-naphthaleneacetic acid), picloram and dicamba are used for somatic embryogenesis (Filippov et al., 2006). The concentration as well as the type of the plant growth regulators in the culture medium also has an important role in the morphogenesis and growth (Shimuzu-Sato et

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al., 2009). Auxin in high concentrations and cytokinin in low concentrations should be included in the culture media generally to increase the callus formation and cellular proliferation (Ge et al., 2006). High concentration of auxin is required for redifferentiation, since mature tissue contains more old cells and more differentiated tissues than immature tissue (Filippov et al., 2006). A major problem with orchardgrass breeding is that the regenerated plant number is too low in all of the the in vitro culture studies. Gene transfer work in this plant has become limited because of this (Cho et al., 2001; Denchev et al., 1997; Tchorbadjieva and Pandchev, 2004; Tomaszewski et al., 1994). The aim of the current study is to investigate the effects of culture media and plant growth regulators on callus induction, multiplication and regeneration efficiency from orchardgrass (*Dactylis glomerata* L.); also to establish an in vitro regeneration system, which is rapid, reliable, repeatable and usable for orchardgrass to improve it as it is an important forage crop.

2. Materials and methods

2.1. Plant material

Mature dry seeds of orchardgrass cultivar Bamba (*Dactylis glomerata*L) were used as a source for endosperm-supported mature embryo (EME).

2.2. Methods

Seeds were surface-sterilized in 70% (v/v) ethanol for 5 min, rinsed twice with sterile distilled water, treated further in commercial bleach (5% sodium hypochlorite) with a few drops of tween for 15 min, and rinsed twice in sterile distilled water. The seeds were keptin sterile water for 16 to 17 h at 4 °C in the dark. In order to prepare the EMEs, shoot axes of mature embryos of orchardgrass were aseptically cut in longitudinal and horizontal directions as seen in Figure 1.

2.2.1. Callus induction

In this study a factorial experiment was performed in a completely randomized design with four replications. Each petri dish was considered as an experimental unit and 10 orchardgrass mature embryos were cultured in each petri dish. The factors consisted of three culture media: MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), N6 (Chu, 1978); three auxin types (2,4-D, dicamba and picloram) and six auxin concentrations (2, 4, 6, 8, 10, and 12 mg/L). All media were solidified with phytagel at 2 g/L, 1.95 g/L MES hydrate, 20 g/L sucrose and adjusted to pH 5.8 with 1 N NaOH. Media solutions containing basal salts and solidifying agent were autoclaved at 121 °C for 20 min for sterilization, vitamins and plant growth regulators were filter-sterilized (0.22-µm porosity). The explants were cultured at 25 °C in dark. After 30 days, callus inductionrate (CI%) was measured in the callus induction medium. CI (%) was formulated as the number of callus formed/number of cultured explants × 100. Then, the developed embryogenic callus was separated from the seeds (Figure 2a).

Figure 1. Cutting area of the seed embryo.

2.2.2 Regeneration of plantlets

After 30 days of culture, all callus was transferred to petri plates containing the regeneration medium, which consisted of MS basic medium, with 20 g/L sucrose and 2 g/L phytagel. Media solidification, pH adjustment, and sterilization were carried out as described for callus induction media. Regeneration callus cultures were incubated in a growth chamber at a 25 ± 1 °C in a 16 h light (62 µmol $m^{-2}s^{-1}$) and 8 h dark photoperiod. Responding embryogenic callus (REC) and regeneration efficiency (RE) were determined after four weeks. REC (%) was calculated asnumber of REC/number of explants × 100, and RE was calculated as the number of regenerated plants/number of REC. Regenerated plantlets were transferred to magenta boxes containing the MS regeneration medium and were grown under the same plant regeneration conditions until they reached 10–12 cm high. Then, the plantlets were transplanted in soil (Figure 2f) and acclimatized.

2.3. Statistical analysis

The study was carried out as 4 replicates and three culture media (MS, SH and N6), three auxin types (2,4-D, dicamba and picloram) and six auxin concentrations (2, 4, 6, 8, 10, and 12 mg/L) with approximately 10 embryos per petri dish. All data for each treatment were considered as continuous variables. Analysis of variance (ANOVA) and Duncan multiple ($p < 0.01$) comparison test were computed with SPSS statistical analysis program (Version 20). In addition, the coefficient of variation for each treatment was performed in SAS (SAS Institute, Cary, NC, USA).

3. Results

3.1. Callus induction rate (CI) (%)

The callus induction value induced by the callus initiation culture medium containing endosperm supported mature embryo is shown in Figures 2a and 2b.

CI rate from endosperm supported mature embryo started very late, only seven-eight days after culture. The results of ANOVA are shown in Table 1. The callus

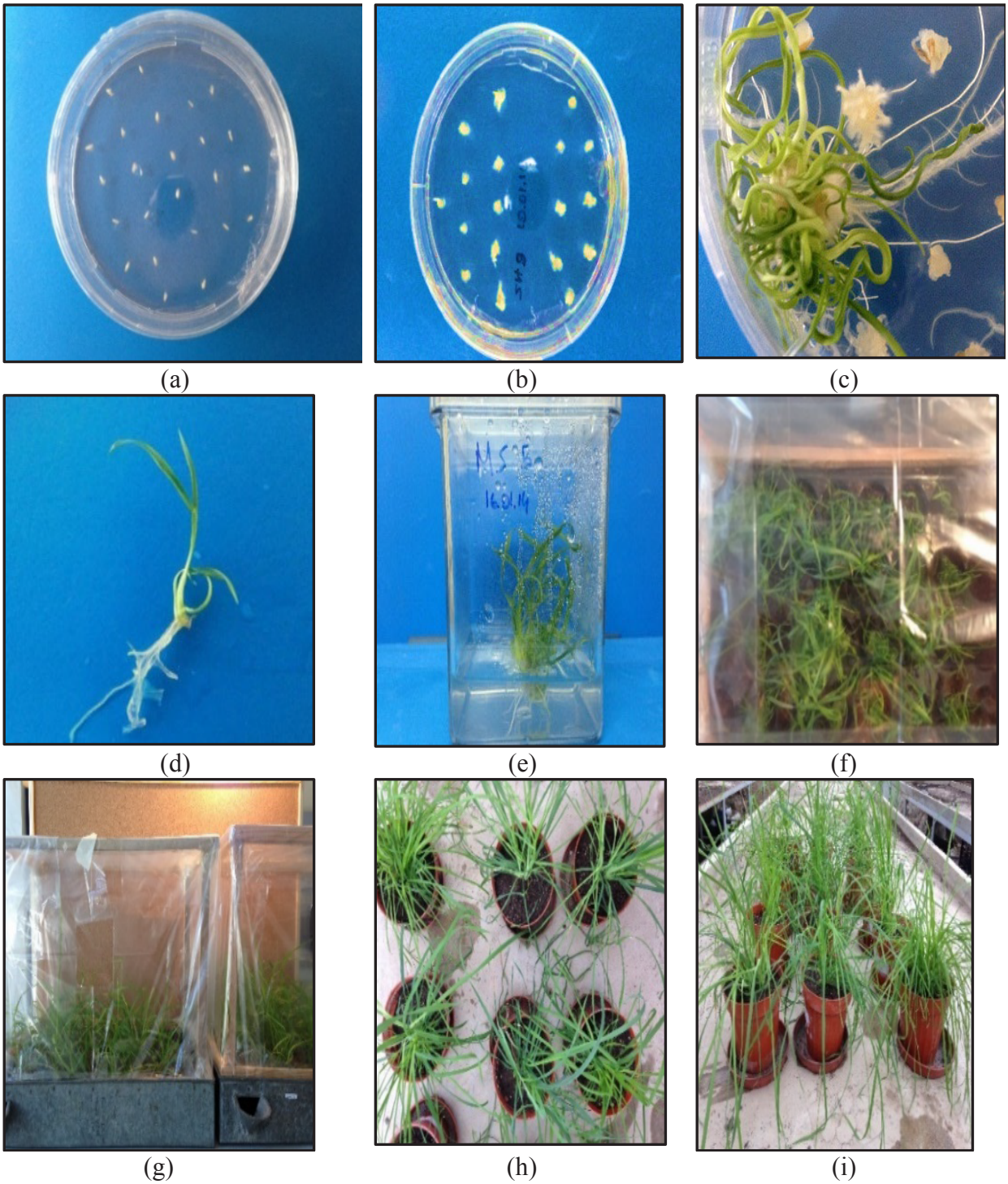

Figure 2. Regenerated plants from tissue culture media; a) starting the culture, b) callus induction, c) regeneration plants occurring from the embryonic calluses, d) regenerated plant, e) development of the roots and shoots in the magenta box of the regenerated plants, f) acclimation of the regenerated plants, g) development of the regenerated plants in viols, h and i) transfer of the regenerated plants to the pots.

induction rate ranged from 39.20% (SH) to 44.20% (N6) depending on the culture media (Table 2a). Dicamba (48.70%) was detected as the highest than 2.4-D (40.80%) for the CI rate (Table 2b). Auxin concentrations resulted in significant differences in the CI rate, with the highest means being obtained from 12 and (47.80%) and the

Table 1. Analysis of variance for different culture media, auxin types, and their concentrations from endosperm supported mature embryo-derived calli of orchardgrass (*Dactylis glomerata* L.).

Source	df	CI (%)		REC (%)		RE (number)	
		F statistic	p-value	F statistic	p-value	F statistic	p-value
Culture media (M)	2	29.11	<.0001	139.86	0.0012	276.72	<.0001
Auxin (A)	2	184.91	<.0001	14.95	<.0001	420.45	<.0001
Concentration (C)	5	59.71	<.0001	43.28	<.0001	114.92	<.0001
M × A	4	29.38	<.0001	34.27	0.0017	83.61	<.0001
M × C	10	2.53	<.00074	4.37	0.0008	12.27	<.0001
A × C	10	27.35	<.0001	7.19	<.0001	37.99	<.0001
M × A × C	20	2.37	<.0016	13.66	<.0001	23.88	<.0001
Error	162						
Total	215						
CV (%)[1]		9.56		25.84		19.08	

** Significant at $p \leq 0.01$; [1]CV: Coefficient of variation.

Table 2a. Mean comparison of different culture media responded embryogenic callus rate (REC) (%) and regeneration efficiency (RE) (number) from endosperm supported mature embryo-derived calli of orchardgrass (*Dactylis glomerata* L.).

Culture media	**CI (%)**	**REC (%)**	**RE (number)**
MS	42.10± 0.97^{B*}	7.90± 039^{A}	6.20± 0.43^{A}
N6	44.20± 1.40^{A}	3.80± 0.36^{C}	2.90 ± 0.42^{B}
SH	39.20± 1.07^{C}	5.70 ± 0.35^{B}	6.10 ±0.39^{A}

*The means in a column with the same letter are statistically indistinguishable from each other (p<0.01).

Table 2b. Mean comparison of different auxin types responded embryogenic callus rate (REC) (%) and regeneration efficiency (RE) (number) from endosperm supported mature embryo-derived calli of orchardgrass (*Dactylis glomerata* L.).

Auxin type	CI (%)	REC (%)	RE (number)
2,4-D	40.80 ± 085^{B*}	6.20 ± 0.49^{A}	6.90 ± 0.52^{A}
Dicamba	48.70 ± 1.06^{A}	6.20 ± 0.32^{A}	5.80 ± 0.37^{B}
Picloram	36.00 ± 1.10^{C}	5.00 ± 0.40^{B}	2.40 ± 0.23^{C}

*The means in a column with the same letter are statistically indistinguishable from each other (p < 0.01).

lowest CI (42.20%) being observed in the concentration of 6 mg/L (Table 2c).

ANOVA exposed significant two-way interactions between culture media × auxin type, culture media × auxin concentration and auxin regulators × auxin concentration were significant ($p \leq 0.01$) in terms of the CI rate (Table 1). According to the interaction effects of culture media and auxin type, the highest CI rate was obtained from the callus initiation medium containing MS medium and dicamba (54.20%) while the lowest was obtained from the MS medium containing picloram (Table 3a). A significant interaction was detected between culture media and auxin concentration in relation to the CI rate (Table 1), indicating that the effect of the former depended on the latter. The highest CI rate (50.80%) was found in the callus initiation N6 culture medium supplemented with maltose 12 mg/L auxin, and the lowest CI rate (29.60%) was detected in the SH culture medium containing sucrose at 2 mg/L auxin concentration (Table 2b). The CI rate was strongly affected by auxin type and concentration. When the effect of interaction between these two parameters on the CI rate was evaluated, the best result (61.30%) was obtained from the culture medium supplemented with 12 mg/L dicamba (Table 3c).

A significant interaction between culture media, auxin type and auxin concentrations were observed at the 0.01 level (Table 1). The highest CI (62.50%) was recorded in the N6 medium with 12 mg/L dicamba, and the lowest value (22.50%) was detected in the SH medium containing 2 mg/L picloram (Table 4, Figure 2b).

Table 2c. Effects of different auxin concentration on callus induction (CI) (%), responding embryogenic callus induction (RECI) (%), regeneration efficiency (RE) (number) from orchardgrass (*Dactylis glomerata* L.).

Concentration (mg/L)	CI (%)	REC (%)	RE (number)
2	$33.30 \pm 1.35^{E*}$	3.30 ± 0.39^{D}	2.90 ± 0.50^{F}
4	38.80 ± 1.31^{D}	4.40 ± 0.47^{C}	3.60 ± 0.52^{E}
6	42.20 ± 1.32^{C}	5.80 ± 0.61^{B}	4.70 ± 0.59^{D}
8	45.40 ± 1.46^{B}	7.50 ± 0.58^{A}	7.50 ± 0.78^{A}
10	43.50 ± 1.34^{C}	6.30 ± 0.54^{B}	6.30 ± 0.53^{B}
12	47.80 ± 2.02^{A}	7.40 ± 0.61^{A}	5.40 ± 0.56^{C}

*The means in a column with the same letter are statistically indistinguishable from each other ($p < 0.01$).

3.2. Responding embryogenic callus induction (REC) (%)

In the present study the embryogenic callus induction from endosperm supported mature embryo started 25–30 days after culture in the respective medium for each treatment. Plant regeneration may not occur in all somatic embryos on embryogenic callus. REC was determined based on the presence of embryogenic callus forming plantlets with healthy roots and shoots (Figure 2c).

The results of ANOVA (Table 1) showed significant effects of culture medium, auxin type, auxin concentration, and their two-way and three-way interactions on the REC rate. MS culture medium resulted in a better medium for REC among the tested three culture media (Table 2a). In terms of the culture medium, the highest REC rate from EME was found in the MS medium (7.90%), and the lowest value (3.80%) was observed in the N6 culture medium (Table 2b). When the main effects of auxin types compared, 2.4-D and dicamba have the highest REC (6.20%). Auxin concentrations lead to significant differences in the REC rate. While the highest REC value (7.40%) was obtained from 12 mg/L, the lowest REC value (3.30%) was observed in the concentration of 2 mg/L (Table 2c). Similar to CI rate, the REC rate was raised with the increase in the concentration of auxin.

The interaction effects of culture medium and auxin type on REC rate were highest in the MS media containing 2,4-D. As shown in Table 3a, it was observed that the REC rate was the lowest in the N6 culture medium supplemented with 2,4-D. The results on the interaction effects on culture medium and auxin concentration showed that the REC rate was highest (9.60%) in the MS medium containing 8 mg/L auxin concentration (Table 3b). Based on effects of auxin type and auxin concentration indicated that the REC rate was highest (44.17%) in the MS media containing maltose and 8mg/L auxin. In addition, the effect of interaction between auxin type and auxin concentration on the REC rate (Table 3c) was highest (10.00%) in the medium with 12 mg/L picloram.

When all the factors were assessed in terms of their effect on REC rate, the maximum REC rate was obtained as 15% from EME cultured in the MS culture media including 8 mg/L 2,4- D auxin type (Table 4, Figure 2c).

3.3. Regeneration efficiency (RE)

In the present study, the effects of culture media, auxin type and auxin concentration, as well as their two-way and three-way interactions on RE were also investigated (Table 1). It

Table 3a. Interaction between culture media and auxin type of callus induction (CI) (%), responding embryogenic callus induction (RECI) (%), regeneration efficiency (RE) (number) from orchardgrass (*Dactylis glomerata* L.).

Culture media	Auxin	CI (%)	REC (%)	RE (number)
MS	2,4-D	$40.0 \pm 1.83^{b*}$	10.6 ± 0.69^{a}	9.5 ± 0.79^{a}
	Dicamba	54.2 ± 1.19^{a}	7.1 ± 0.51^{b}	5.1 ± 0.43^{b}
	Picloram	32.1 ± 1.59^{c}	6.0 ± 0.42^{b}	3.9 ± 0.39^{c}
N6	2,4-D	42.3 ± 1.31^{b}	2.5 ± 0.52^{c}	3.9 ± 1.08^{a}
	Dicamba	48.3 ± 1.63^{a}	5.0 ± 0.60^{b}	3.6 ± 0.48^{a}
	Picloram	42.1 ± 1.80^{b}	3.8 ± 0.62^{b}	1.1 ± 0.19^{b}
SH	2,4-D	40.2 ± 1.25^{b}	5.4 ± 0.28^{b}	7.3 ± 0.28^{b}
	Dicamba	43.5 ± 1.96^{a}	6.5 ± 0.47^{a}	8.8 ± 0.49^{a}
	Picloram	33.8 ± 1.73^{c}	5.2 ± 0.92^{b}	2.3 ± 0.37^{c}

*The means in a column with the same letter are statistically indistinguishable from each other ($p < 0.01$).

Table 3b. Interaction between culture media and concentration on callus induction (CI) (%), responding embryogenic callus induction (RECI) (%), regeneration efficiency (RE) (number) from orchardgrass (*Dactylis glomerata* L.).

Culture media	Concentration (mg/L)	CI (%)	REC (%)	RE (number)
MS	2	33.7 ± 3.08^{d*}	5.0 ± 00^{d}	4.2 ± 1.01^{c}
	4	40.8 ± 3.36^{c}	6.7 ± 0.71^{c}	4.8 ± 0.86^{c}
	6	42.1 ± 3.04^{bc}	9.2 ± 1.20^{ab}	6.0 ± 1.06^{b}
	8	46.7 ± 3.65^{a}	9.6 ± 1.29^{a}	9.4 ± 1.68^{a}
	10	43.8 ± 3.08^{abc}	7.9 ± 0.74^{bc}	6.1 ± 021^{b}
	12	45.4 ± 3.66^{ab}	9.2 ± 0.56^{ab}	6.6 ± 0.20^{b}
N6	2	36.7 ± 1.66^{c}	1.7 ± 0.71^{d}	0.7 ± 0.29^{d}
	4	37.9 ± 1.68^{c}	3.3 ± 0.71^{c}	1.3 ± 0.32^{cd}
	6	45.8 ± 1.20^{b}	3.3 ± 0.71^{c}	1.5 ± 0.37^{c}
	8	47.9 ± 0.74^{ab}	5.8 ± 0.56^{a}	6.0 ± 1.35^{a}
	10	46.3 ± 2.05^{b}	3.3 ± 0.71^{c}	5.4 ± 1.31^{a}
	12	50.8 ± 3.10^{a}	5.0 ± 1.23^{b}	2.3 ± 079^{b}
SH	2	29.6 ± 1.68^{d}	3.3 ± 0.71^{c}	3.8 ± 0.82^{b}
	4	37.5 ± 1.30^{c}	3.3 ± 0.71^{c}	4.6 ± 1.03^{b}
	6	38.8 ± 1.95^{bc}	5.0 ± 00^{b}	6.5 ± 0.80^{a}
	8	41.7 ± 2.16^{b}	7.1 ± 0.74^{a}	7.2 ± 0.84^{a}
	10	40.4 ± 1.43^{bc}	7.5 ± 0.75^{a}	7.4 ± 0.89^{a}
	12	47.1 ± 3.76^{a}	7.9 ± 0.96^{a}	7.3 ± 10.5^{a}

*The average shown with the same letter are statistically indistinguishable from each other ($p < 0.01$). *The means in a column with the same letter are statistically indistinguishable from each other ($p < 0.01$).

was observed that among the culture media, MS culture medium (6.20 number) resulted in higher RE than the other culture media (Table 2a). RE significantly depended on the auxin type with the highest value (0.85) being achieved in calli formed in the medium supplemented with 2.4-D, followed by the media containing dicamba and picloram (Table 2b). In addition, RE increased up to 8 mg/L, but after this concentration, RE number was decreased (Table 2c).

The effect of culture medium varied depending on the auxin type, as well as the auxin concentration source. The highest RE (9.60) was found in calli cultured in the MS medium containing 2.4-D (Table 3a). The effect of culture medium on RE varied according to the auxin concentrations used in the callus initiation medium. The highest RE was recorded in the MS medium containing 8 mg/L auxin (9.4 number), as opposed to this the lowest RE was determined in 2 mg/L N6 culture media (0.70 number) (Table 3b). The highest RE was occurred in culture medium containing 2.4-D with 8 mg/L auxin (12.50 number), while the lowest average RE (1.10 number) was obtained with picloram containing 2 mg/L (Table 2c).

It was observed that culture medium, auxin type, and auxin concentration played an important role in RE. The maximum RE (17.0 number) occurred in calli formed in the MS medium containing 8 mg/L 2.4-D (Table 4). All acclimated plants developed into normal plants in the greenhouse and set seed (Figures 2d and 2i).

4. Discussion

Genetic engineering studies depend on an efficient plant regeneration system (Aydin et al., 2016). Mature embryos are alternative explant resources in genetic transformation studies (Aydin et al., 2011). Conger and Carabia (1978) and Sahin et al. (2010) have also used mature embryos for regeneration in orchardgrass, but these researchers have attained too few regenerated plants in their studies. Therefore, mature embryos are not often used because their regeneration capacities are low with the somatic embryogenesis method.

In the current study, the mature embryos of the orchardgrass were cultured for regeneration in three culture media with three auxins types at six concentrations.

Table 3c. Interaction between auxin type and concentration on callus induction (CI) (%), responding embryogenic callus induction (RECI) (%), regeneration efficiency (RE) (number) from orchardgrass (*Dactylis glomerata* L.).

Auxin	Concentration (mg/L)	CI (%)	REC (%)	RE (number)
2,4-D	2	35.4 ± 1.29^{de*}	3.3 ± 0.71^{d}	4.8 ± 1.11^{de}
	4	40.8 ± 0.83^{bc}	5.0 ± 1.23^{c}	5.4 ± 1.18^{cd}
	6	44.2 ± 1.35^{b}	6.3 ± 1.75^{b}	6.2 ± 1.40^{c}
	8	51.3 ± 1.08^{a}	9.2 ± 1.35^{a}	12.5 ± 1.07^{a}
	10	38.8 ± 1.52^{cd}	6.7 ± 0.71^{b}	7.9 ± 0.61^{b}
	12	34.6 ± 1.78^{e}	6.7 ± 0.71^{b}	4.6 ± 0.79^{e}
Dicamba	2	39.6 ± 1.99^{e}	3.3 ± 0.71^{d}	2.7 ± 0.73^{e}
	4	45.0 ± 2.13^{d}	5.0 ± 00^{c}	4.0 ± 0.55^{d}
	6	46.7 ± 2.07^{cd}	5.8 ± 0.56^{bc}	5.3 ± 0.80^{c}
	8	48.8 ± 1.64^{bc}	6.3 ± 0.65^{bc}	6.7 ± 0.81^{b}
	10	50.8 ± 1.72^{b}	6.7 ± 0.71^{b}	7.8 ± 0.68^{a}
	12	61.3 ± 1.08^{a}	10.0 ± 00^{a}	8.3 ± 0.76^{a}
Picloram	2	25.0 ± 1.23^{e}	3.3 ± 0.71^{b}	1.1 ± 0.25^{c}
	4	30.4 ± 0.96^{d}	3.3 ± 0.71^{b}	1.3 ± 0.37^{c}
	6	35.8 ± 2.20^{c}	5.4 ± 0.41^{a}	2.5 ± 0.26^{b}
	8	36.3 ± 2.22^{c}	7.1 ± 0.74^{a}	3.3 ± 0.47^{a}
	10	40.8 ± 2.11^{b}	5.4 ± 1.29^{a}	3.1 ± 0.71^{ab}
	12	47.5 ± 1.56^{a}	5.4 ± 1.43^{a}	3.3 ± 0.77^{a}

*The means in a column with the same letter are statistically indistinguishable from each other ($p < 0.01$).

Successful plant regeneration was obtained from the endosperm supported mature embryo in several treatment combinations. Endosperm supported mature embryos are better in terms of plant regeneration when compared to direct embryos because endosperm provides better nutrition than the artificial culture media (Bartok and Sagi, 1990). Another view is that some cells in the embryo could receive some type of signals from the endosperm that would improve differentiation of the explant and as a result, the regenerated plants will occur (Chen et al., 2006).

Plant growth regulators were tested due to their impacts such as the stimulation for a complete embryo formation, callus (undifferentiated cell group) formation and offspring formation; and their impacts have started to be examined. The most widely used auxin types for embryogenesisare 2,4-D and naphthalene acetic acid (NAA), picloram and dicamba (Gaspar et al., 1996). It has been determined in several studies that the best auxin type for callus induction is dicamba and 2,4-D for responding embryogenic callus and regeneration activity (Hossein Pour et al., 2019). For wheat, in the studies in which auxin types have been compared in the endosperm supported and nonsupported mature embryo cultures, dicamba is more efficient than 2,4-D (Mendoza and Kaepler, 2002; Filippov et al., 2006). For crested wheat grass, the best callus formation was obtained using dicamba (Can et al., 2008). Papenfuss and Carman (1987) stated that dicamba is more rapidly used in plant metabolism and therefore it increases shoot formation. However, in the study conducted by Redway et al. (1990) in wheat tissue culture, no difference was found between dicamba and 2,4-D.

The amounts as well as the type of the plant growth regulators in the culture media have a significant role in morphogenesis and growth. Usually in regeneration protocols, auxins are required in high concentrations and cytokinins in low concentrations to increase callus formation and cellular proliferation (Ge et al., 2006). In the current study, when the auxin type, concentration and culture media are assessed together, the highest regenerated plant number has been attained from MS medium containing 8 mg/L 2,4-D. Many researchers have found that 2,4-D is successfully used for callus formation (Ozgen et al., 1998; Ozgen et al., 1996). Chen et al (2006) used MS medium containing 8 mg/L 2,4-D

Table 4. Interaction among culture medium, auxin type and concentration on callus induction (CI) (%), responding embryogenic callus induction (RECI) (%), regeneration efficiency (RE) (number) from orchardgrass (*Dactylis glomerata* L.).

Culture media	Auxin	Concentration (mg/L)	CI (%)	REC (%)	RE (number)
MS	2,4-D	2.0	32.5 ± 1.44^{c*}	5.0 ± 0.00^{c}	8.7 ± 0.67^{c}
		4.0	41.3 ± 1.25^{b}	10.0 ± 0.00^{b}	8.8 ± 0.13^{c}
		6.0	41.3 ± 3.14^{b}	13.8 ± 1.25^{a}	11.0 ± 0.40^{b}
		8.0	55.0 ± 2.04^{a}	15.0 ± 0.00^{a}	17.0 ± 0.70^{a}
		10.0	37.5 ± 3.22^{bc}	10.0 ± 0.00^{b}	6.0 ± 0.00^{d}
		12.0	32.5 ± 3.22^{c}	10.0 ± 0.00^{b}	6.1 ± 0.31^{d}
	Dicamba	2.0	46.3 ± 2.39^{b}	5.0 ± 0.00^{b}	2.5 ± 0.64^{c}
		4.0	53.8 ± 2.39^{a}	5.0 ± 0.00^{b}	3.0 ± 0.40^{bc}
		6.0	53.8 ± 2.39^{a}	7.5 ± 1.44^{ab}	4.0 ± 0.40^{b}
		8.0	55.0 ± 2.04^{a}	7.5 ± 1.44^{ab}	6.7 ± 0.47^{a}
		10.0	56.3 ± 2.39^{a}	7.5 ± 1.44^{ab}	7.0 ± 0.00^{a}
		12.0	60.0 ± 2.04^{a}	10.0 ± 0.00^{a}	7.2 ± 0.00^{a}
	Picloram	2.0	22.5 ± 1.44^{d}	5.0 ± 0.00^{a}	1.3 ± 0.10^{d}
		4.0	27.5 ±1.44^{cd}	5.0 ± 0.00^{a}	2.8 ± 0.47^{c}
		6.0	31.3 ±1.25^{c}	6.3 ± 1.25^{a}	3.0 ± 0.40^{c}
		8.0	30.0 ± 0.00^{c}	6.3 ± 1.25^{a}	4.5 ± 0.97^{b}
		10.0	37.5 ± 3.22^{b}	6.3 ± 1.25^{a}	5.3 ± 0.19^{ab}
		12.0	43.8 ± 2.39^{a}	7.5 ± 1.44^{a}	6.3 ± 0.25^{a}
N6	2,4-D	2.0	40.0 ± 2.04^{b}	0.0 ± 00	0.0 ± 00^{c}
		4.0	40.0 ± 2.04^{b}	0.0 ± 00	0.0 ± 00^{c}
		6.0	46.3 ±2.39^{ab}	0.0 ± 00	0.0 ± 00^{c}
		8.0	50.0 ± 0.00^{a}	5.0 ± 0.00	11.8 ± 0.62^{a}
		10.0	38.8 ± 3.75^{b}	5.0 ± 00	10.5 ± 00^{b}
		12.0	38.8 ± 3.75^{b}	5.0 ± 00	1.0 ± 00^{c}
	Dicamba	2.0	40.0 ± 204^{d}	0.0 ± 00	0.0 ± 00^{c}
		4.0	42.5 ± 1.44^{cd}	5.0 ± 00	2.6 ± 0.75^{b}
		6.0	46.3 ± 2.39^{bc}	5.0 ± 00	3.0 ± 0.00^{b}
		8.0	47.5 ± 1.44^{bc}	5.0 ± 00	4.3 ± 1.52^{ab}
		10.0	51.3 ± 1.25^{b}	5.0 ± 00	5.8 ± 0.47^{a}
		12.0	62.5 ± 1.44^{a}	10.0 ± 00	6.0 ± 00^{a}
	Picloram	2.0	30.0 ± 00^{c}	5.0 ± 0.00^{b}	2.0 ± 0.20^{a}
		4.0	31.3 ± 1.25^{c}	5.0 ± 0.00^{b}	1.3 ± 0.25^{a}
		6.0	45.0 ± 2.04^{b}	5.0 ± 0.00^{b}	1.5 ± 0.28^{a}
		8.0	46.3 ± 1.25^{b}	7.5 ± 1.44^{a}	2.0 ± 0.40^{a}
		10.0	48.8 ± 1.25^{ab}	0.0 ± 00^{c}	0.0 ± 00^{b}
		12.0	51.3 ± 1.25^{a}	0.0 ± 0.00^{c}	0.0 ± 0.00^{b}

Table 4. (Continued).

SH	2,4-D	2.0	33.8 ± 1.25^{d}	5.0 ± 0.00^{b}	5.8 ± 0.47^{b}
		4.0	41.3 ± 1.25^{c}	5.0 ± 00^{b}	7.5 ± 0.73^{ab}
		6.0	45.0 ± 0.00^{b}	5.0 ± 0.00^{b}	7.5 ± 0.64^{ab}
		8.0	48.8 ± 1.25^{a}	7.5 ± 1.44^{a}	8.8 ± 047^{a}
		10.0	40.0 ± 0.00^{c}	5.0 ± 0.00^{b}	7.3 ± 0.62^{ab}
		12.0	32.5 ± 1.44^{c}	5.0 ± 0.00^{b}	6.7 ± 0.62^{b}
	Dicamba	2.0	32.5 ± 1.44^{d}	5.0 ± 0.00^{b}	5.7 ± 0.23^{d}
		4.0	38.8 ± 1.25^{c}	5.0 ± 0.00^{b}	6.3 ± 0.62^{d}
		6.0	40.0 ± 2.04^{bc}	5.0 ± 0.00^{b}	8.9 ± 0.59^{c}
		8.0	43.8 ± 1.25^{bc}	6.3 ± 1.25^{b}	9.3 ± 0.75^{bc}
		10.0	45.0 ± 2.04^{b}	7.5 ± 1.44^{b}	10.8 ± 0.59^{ab}
		12.0	61.3 ± 2.39^{a}	10.0 ± 0.00^{a}	11.7 ± 0.62^{a}
	Picloram	2.0	22.5 ± 1.44^{c}	0.0 ± 00^{c}	0.0 ± 00^{b}
		4.0	32.5 ± 1.44^{b}	0.0 ± 00^{c}	0.0 ± 00^{b}
		6.0	31.3 ± 2.39^{b}	5.0 ± 0.00^{b}	3.0 ± 0.00^{a}
		8.0	32.5 ± 1.44^{b}	7.5 ± 1.44^{ab}	3.5 ± 0.61^{a}
		10.0	36.3 ± 2.39^{b}	10.0 ± 0.00^{a}	4.0 ± 0.70^{a}
		12.0	47.5 ± 3.22^{a}	8.8 ± 2.39^{a}	3.50 ± 0.28^{a}

*The means for each auxin in a column with the same letter are statistically indistinguishable from each other ($p < 0.01$).

in the endosperm supported mature embryo culture for wheat. Ahmet and Adak (2007) working on wheat mature embryo culture cultured mature embryos in MS medium containing 8 mg/L 2,4-D. Birsin and Ozgen (2004) working in triticale suggested MS medium containing 8 mg/L 2,4-D for endosperm supported mature embryo culture. However, Filippov et al. (2006) found that mature embryos are older and contain more differentiated tissues than the nonmature embryos and for this reason, higher ratios of auxin should be used for dedifferentiation. Moreover, the same researchers have stated that endosperm may absorb the plant growth regulators in the endosperm supported mature embryo culture and therefore, higher ratios of auxin should be used than the endosperm nonsupported mature embryo culture.

Plant regeneration in higher concentrations has been determined to be better in three auxin types used in this study. The highest plant regeneration occurs at 8 mg/L for 2,4-D, and with 12 mg/L for dicamba and picloram. Filippov et al. (2006) working on mature embryos in the wheat attained the best plant regeneration from 10 mg/L of 2,4- D and 12 mg/L of dicamba. In the same study, the plant regeneration in the media containing 14 mg/L dicamba was higher than the media containing 10 mg/L 2,4-D. Muhumuza and Okori (2013) attained the best plant regeneration in sorghum species with 2.5 mg/L 2,4-D and 0.5 mg/L kinetin and also found that the use of 2,4-D in high ratio decreases the callus formation and plant regeneration. Increasing concentrations of 2,4-D were specified by McDonnell and Conger (1984) to decrease leaf development in bluegrass and the callus development is high in the medium without 2,4-D and with 5, 10, 20 µM dicamba. These results show that dicamba may be more efficient in high concentrations when compared to 2,4-D. It could distinctly remain in high concentrations in the plant cells (Moore, 1989). Abnormalities and somaclonal variation are known to increase in the higher concentrations of 2,4-D and for this reason, the auxins such as dicamba and picloram could be used as an alternative to 2,4-D (Pedrosa and Vasil, 1996). However, it is necessary to choose the best auxin type and concentration since somaclonal variation is not desired in gene transfer studies. Studies regarding somaclonal variation will be needed as a continuation of the current study.

One of the factors affecting the success in tissue culture is the main culture medium. Although there are many culture media formulations specified by various researchers, MS, B5, LS, N6, and SH are the most commonly used culture media. MS medium variations are successfully used in many plant species. In this study, SH medium produced better results than other media (MS and N6). SH is effective for both monocotyledon and

dicotyledon plants. Can et al. (2008) in a study on crested wheatgrass, found the highest shoot formation in SH medium. However, Ahn et al. (1985) have expressed that the callus development was better in N6 when compared to MS for *Lolium multiflorum*, *Festuca rubra* and *Dactylis glomerata*. In the study conducted by Rim et al. (2000) on Italian ryegrass (*Lolium multiflorum*) plant, MS was the most efficient nutrient medium for plant regeneration among the media of SH, MS and N6.

Consequently, the current study determined that endosperm supported mature embryos could be successfully used in orchardgrass for an in vitro regeneration system that is rapid, reliable, repeatable and usable in modern plant improvement.

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Informed consent
This article does not contain any studies with human participants or animals performed by any of the authors.

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