DEVELOPMENT OF AN EFFICIENT REGENERATION SYSTEM VIA SOMATIC EMBRYOGENESIS OBTAINED FROM MATURE EMBRYOS IN SOME GRAIN AND SILAGE SORGHUM CULTIVARS

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Abstract. An effective regeneration system was developed from embryonic callus that was formed by using mature embryos in 6 sorghum cultivars (Gözde 80, Greengo, Leoti, Beydarı, Aldarı and Akdarı). Different auxin (2,4-D and 2,4,5-T) and cytokinin (zeatin and kinetin) combinations on somatic embryogenesis were studied. The highest embryonic callus in all cultivars was derived from cultures in MS medium containing 1 mg/l 2,4-D. The transfer of embryonic callus obtained from medium containing 2,4,5-T + kinetin to the shooting medium (1 or 2 mg/l BA +1.5 mg/l TDZ +1 mg/l IAA) and subsequently rooting medium (½ MS with 1 mg/l NAA) resulted in a higher shooting and rooting. Different concentrations of BA in the shooting medium did not affect shoot formation. Akdarı and Greengo cultivars produced better callus induction and regeneration than the other cultivars as grain and silage types, respectively. Rooting and surviving rates varied between 10.55-68.37% depending on the growth regulators used at the beginning of culture. Growth and survival rates were increased in plants transferred from high-shoot-rate cultivars to the rooting medium.
Keywords: *sorghum, embryonic callus, 2,4-D, 2,4,5-T, kinetin*

Introduction

Sorghum (*Sorghum bicolor* L. Moench.) is an important cereal like rice, barley, wheat and maize around the World (Ritter et al., 2007; Motlhaodi et al., 2014; Sinha and Kumaravadivel, 2016). It is originated from North Africa and widely grown in tropical and subtropical regions (Dillon et al., 2007). Sorghum is widely used as a food source for human and animal nutrition in the arid and semi-arid regions (Sing and Sing, 1992; Sharma and Ortiz, 2000).

Various reasons such as physiological, morphological and genetic diversity, a wide range of genetic stocks, maps and application of versatile tests through self-fertilization make sorghum important in terms of agriculture, physiological and biotechnological studies (Kong et al., 2000; Hart et al., 2001; Zongo et al., 1993). In cereals, genetic studies performed by gene transfer methods and other biotechnological developments are dependent on the establishment of an effective and repeatable plant regeneration system.

It has been reported that immature embryos are the best explant source in monocots species (Tiidema and Truve, 2004). However, several factors restrict the use of immature embryos for in vitro culture of sorghum; difficulties of donor plants maintenance, problems with isolation and sterilization of immature embryos, unequal production of embryonic callus by different genotypes, and the tendency of good friable embryogenic callus producing genotypes to secrete polyphenols or other inhibitory substances (Rao et al., 1995; Seetharama et al., 2000; Pola et al., 2009).

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Although the mature embryos come to the forefront with an unlimited explant source throughout the year, the embryonic callus from mature seeds are difficult and the lack of study performed in cultivated sorghum genotypes is noteworthy. In the previous studies, 2,4-D (2,4-Dichlorophenoxyacetic acid) combined with kinetin provided embryonic callus and regeneration in different sorghum genotypes (Mackinnon et al., 1986; Nirwan and Kothari, 2004; Zhao et al., 2010; Zarif et al., 2013; Hassan et al., 2014). Also, Pola and Mani (2006) stated that 2,4,5-T (2,4,5-Trichlorophenoxyacetic acid) gave the effective embryogenic callus induction from the leaf of sorghum. Pola et al. (2009) investigated that the effect of 2,4-D and 2,4,5-T alone on plant regeneration using mature embryo of sorghum. In this study, the effect of 2,4-D and 2,4,5-T combined with kinetin and zeatin were observed on embryogenic callus induction and regeneration in the mature embryos in grain and silage types of sorghum which have no knowledge about their regeneration capabilities.

Materials and Methods

The study was carried out in Eskişehir Osmangazi University, Faculty of Agriculture, Department of Field Crops, Eskişehir, Turkey. Seeds of Beydarı, Aldarı, Akdarı, Gözde 80, and Leoti cultivars were obtained from the Bati Akdeniz Agricultural Research Institute, and Greengo cultivar provided by May Seed Company. While the grain types (Beydarı, Aldarı and Akdarı) have short plant height, the silage types (Greengo, Gözde 80 and Leoti) are tall and they have the possibility of use regrowth. The grain of Akdarı is white and does not include tannin. These seeds were first allowed in 96% ethanol for 4 min and subsequently 30 min in a 30% bleach solution (Domestos commercial bleach; 4,5% Sodium hypochlorite) for surface sterilization. After sterilization, the seeds were rinsed 5 times with distilled water and then were soaked for 22 h in distilled water at room temperature for easy dissection of embryos from seed. Mature embryos were aseptically dissected from seeds under the stereomicroscope and were cultured scutellum upward on solid MS medium (Murashige and Skoog, 1962) containing growth regulators at different concentrations and combinations. The various levels of 2,4-D and 2,4,5-T (1, 2 and 4 mg/l) and their combinations with 0,5 mg/l kinetin and zeatin were used for callus induction. After callus induction, the calli from 1 mg/l were transferred to media with 1,5 mg/l TDZ (Thidiazuron) and 1 mg/l IAA (3-Indoleacetic acid) combined with 1 and 2 mg/l concentrations of BA (6-Benzylaminopurine) for shoot formation (*Table 1*).

Sucrose (30 g/l) was added to medium and the pH was adjusted to 5.8 and the medium was solidified with 6.5% agar. The medium was autoclaved at 121°C and 1,2 atm pressure for 20 min. All the cultures were incubated in a growth chamber (Panasonic MLR-352H-PA) at 25±1°C, 60% humidity and 16h / 8h photoperiod (long day conditions). The experiment was arranged in three factors in completely randomized design (CRD) with 4 replicates and 5 explants were used in each replicate. First factor was sorghum cultivars, second was auxin types and combinations and third was in auxin concentrations for callus induction while BA concentration was inserted as third factor in shooting and rooting. Arcsine ($\sqrt{x}$) transformation was applied in percentages data before statistical analysis (Snedecor and Cochran, 1992). Healthy shoots were rooted on 1/2 MS medium containing 1 mg/l NAA (1-Naphthaleneacetic acid). The rooted plantlets were transferred to pots containing peat and vermiculite (4:1) for acclimatization at climate room.

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Table 1. *The summarise experimental design in a tabular form*

Callus induction (mg/l)				Shootin formation (mg/l)			Rooting (mg/l)
2,4-D	**2,4,5-T**	**Kinetin**	**Zeatin**	**BA**	**TDZ**	**IAA**	**NAA**
1	-	-	-	1	1.5	1	1
	-	-	-	2	1.5	1	1
2	-	-	-				
4	-	-	-				
1	-	0.5	-	1	1.5	1	1
	-	0.5	-	2	1.5	1	1
2	-	0.5	-				
4	-	0.5	-				
1	-	-	0.5	1	1.5	1	1
	-	-	0.5	2	1.5	1	1
2	-	-	0.5				
4	-	-	0.5				
-	1	-	-	1	1.5	1	1
				2	1.5	1	1
-	2	-	-				
-	4	-	-				
-	1	0.5	-	1	1.5	1	1
		0.5	-	2	1.5	1	1
-	2	0.5	-				
-	4	0.5	-				
-	1	-	0.5	1	1.5	1	1
		-	0.5	2	1.5	1	1
-	2	-	0.5				
-	4	-	0.5				

Results and Discussion

Callus induction

The embryogenic tight and compact calli were obtained from mature embryos as well as non-embryogenic weak and soft calli in dark condition after 4 weeks culture initiation (*Figs. 1a* and *1b*). There were significant differences in the main factors (cultivars, auxin types and combinations and their different doses) and their interactions in terms of callus induction percentage at 1% level. The callus induction from mature embryos in sorghum cultivars ranged from 54.82% to 90.58%, in Leoti and Akdarı, respectively (*Table 2*). It is known that success in callus formation and plant regeneration are largely dependent on the genotype in sorghum (Seetharama et al., 2000; Pola, 2011; Zarif et al., 2013; Hassan et al., 2014). The highest callus induction was obtained from pure doses of the auxins compared to their combinations with kinetin and zeatin. However, combinations of 2,4-D induced more callus formation than combinations of 2,4,5-T. Jogeswar et al. (2007) stated that pure 2,4-D has a crucial importance for embryonic callus initiation in monocotyledon plants. Also, Zhao et al. (2010) observed that 2,4-D alone was sufficient to induce callus formation and the additional kinetin in high concentrations decreased the callus induction in sorghum. Increased doses of auxin affected callus formation negatively and it varied between 74.61% and 56.25% (*Table 2*). Zhao et al. (2010) found out callus induction obtained from germinating seed of sorghum increased with increasing concentrations of 2,4-D (4 mg/l) in contrast to our study.

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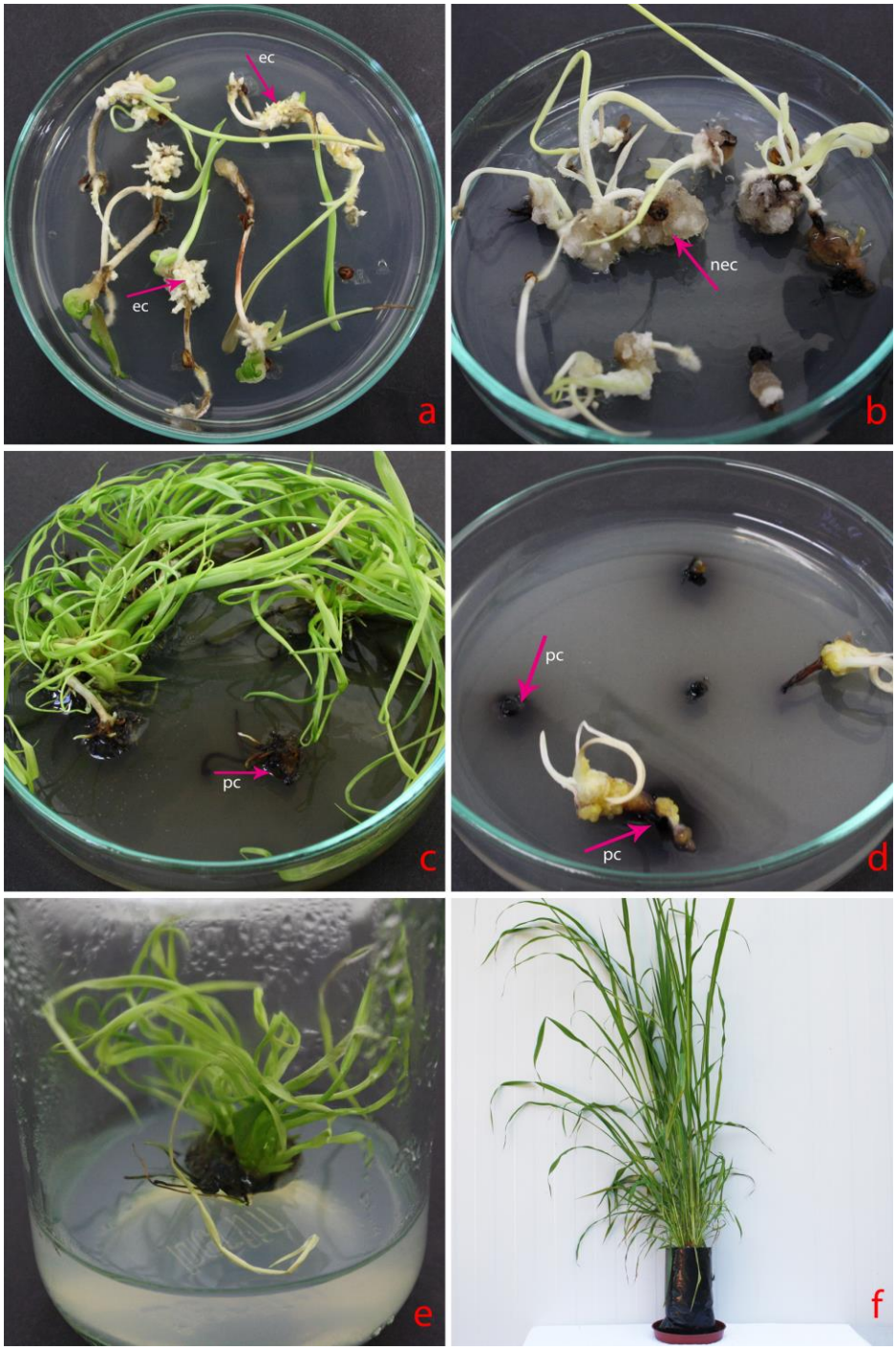

Figure 1. *An efficient regeneration system in grain and silage sorghum cultivars. (a) tight and compact embryogenic calli (ec) of Greengo cultivar on callus induction medium (MS supplemented with 1.0 mg/L 2,4,5-T, 0,5 mg/l kinetin) after four week's culture initiation, (b) weak and soft non-embryogenic calli (nec) of Gözde-80 cultivar on callus induction medium (MS supplemented with 1.0 mg/L 2,4,5-T, 0,5 mg/l kinetin), (c) callus regenerating of Greengo on shoot induction medium (MS supplemented with 1,5 mg/l TDZ, 1 mg/l IAA, 1 mg/l BA) after eight week's initiation and phenolic compound (pc) formation during shoot formation, (d) phenolic compound (pc) formation during callus induction of Aldarı on callus induction medium (MS supplemented with 1.0 mg/L 2,4-D), (e) rooting of Greengo on root induction medium (½ MS supplemented with 1 mg/l NAA), (f) acclimatization of Greengo rooting regenerates*

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In comparison with the study, Zarif et al. (2013) showed that 4 mg/l of 2,4-D was the best concentration in callus induction from seed and immature inflorescence and that the lowest and the highest levels of 2,4-D (1 and 6 mg/l) delayed callus induction. In contrast to these studies, Lu et al. (1983) and Pola et al. (2008) stated that the high 2,4-D concentration in cereals negatively affected the embryonic callus induction in support of our findings. The high-frequency embryogenic calli were also obtained from mature embryos using 2 mg/l of 2,4-D with combined 0.5 mg/l of kinetin by Arulselvi and Krishnaveni (2009).

Table 2. *Mean values of callus induction in sorghum cultivars, auxin types and combinations and their doses*

Cultivars	**Callus induction percentage (%)**
Gözde 80	59.62^{c*}
Greengo	72.11^{b}
Leoti	59.43^{dc}
Beydarı	67.75^{b}
Aldarı	54.82^{d}
Akdarı	90.58^{a}
Auxin types and combinations	
2.4-D	80.08^{a}
2.4-D+Zeatin	69.18^{bc}
2.4-D+Kinetin	68.95^{c}
2.4.5-T	74.88^{b}
2.4.5-T+Zeatin	57.75^{d}
2.4.5-T+Kinetin	53.84^{d}
Doses (mg/l)	
1	74.61^{a}
2	69.89^{b}
4	56.25^{c}

*The means shown similar letters are not statistically different at $p \leq 0.05$

Plant regeneration

The shoots and chlorophyll developed 6 weeks after the initiation of culture, and shoot formation was completed within a total of 8 weeks (*Fig. 1c*). The concentrated phenolic compound formation was observed during both callus and shoot induction depending on the genotypes which were negatively affected at the point of regeneration (*Figs. 1c* and *1d*). Nguyen et al. (2007) stated that the most common problem of sorghum tissue culture is the high rate of secretion of phenolic compounds.

To determine the plant regeneration, embryonic calli obtained from 1 mg/l of 2,4-D and 2,4,5-T and their combinations with 0,5 mg/l kinetin and zeatin were used. The best shoot formation was determined in shoot media containing BA regardless of 1 and 2 mg/l (Data not shown). Setting aside the doses of BA, the effects of the genotypes and hormones on plant regeneration were found to be significant (*Table 3*).

Although Akdarı had a high callus induction percentage, the highest number of shoots (2.73 shoots per callus) was obtained with the Greengo cultivar. Also, 2,4,5-T combined with kinetin showed the highest number of shoots (3.39 shoots per callus), while this combination was not effective in callus induction percentage (*Table 3*). Unlike our study, those of Gupta et al. (2006) and Pola et al. (2008) revealed that there was a significant positive correlation between embryonic callus frequency and the number of shoots of sorghum. Shoot number per callus was clearly increased when kinetin was added to medium with 2,4,5-T. Pola et al. (2008) and Hagio (2002) reported

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that adding cytokinins (kinetin or 6-benzyladenine) to the callus induction medium resulted in the highest regeneration rate; this supported our findings. Additionally, the use of regeneration medium without growth regulators gave the next highest regeneration rate. Zarif et al. (2013) showed that the addition of kinetin to the culture medium had no effect on the regeneration of sorghum.

Table 3. *Mean values of shoot formation in sorghum cultivars, auxin types and combinations*

Cultivars	Shoot numbers per callus (number)
Gözde 80	1.52^{b*}
Greengo	2.73^{a}
Leoti	1.95^{b}
Beydarı	1.50^{b}
Aldarı	1.13^{b}
Akdarı	1.53^{b}
Auxin types and combinations	
2.4-D	1.82^{b}
2.4-D+Zeatin	0.73^{c}
2.4-D+Kinetin	1.28^{bc}
2.4.5-T	2.00^{b}
2.4.5-T+Zeatin	1.40^{bc}
2.4.5-T+Kinetin	3.39^{a}

*The means shown similar letters are not statistically different at p≤0.05

Rooting

The regenerates obtained from the shooting medium were successfully rooted when transferred to the rooting medium containing 1 mg/l NAA (*Fig. 1e*). Also, many previous studies have shown that NAA is successful in the rooting of sorghum (Pola and Mani, 2006; Sai Kishore et al., 2006; Pola et al., 2008). These rooted plants were transferred to a pot containing peat and vermiculite (4:1) and were successfully acclimated to outdoor condition at climate room (*Fig. 1f*). While BA concentration did not affect rooting (data not shown), the rooting percentage of sorghum cultivars and auxin types and combinations showed in significant differences (*Fig. 2*).

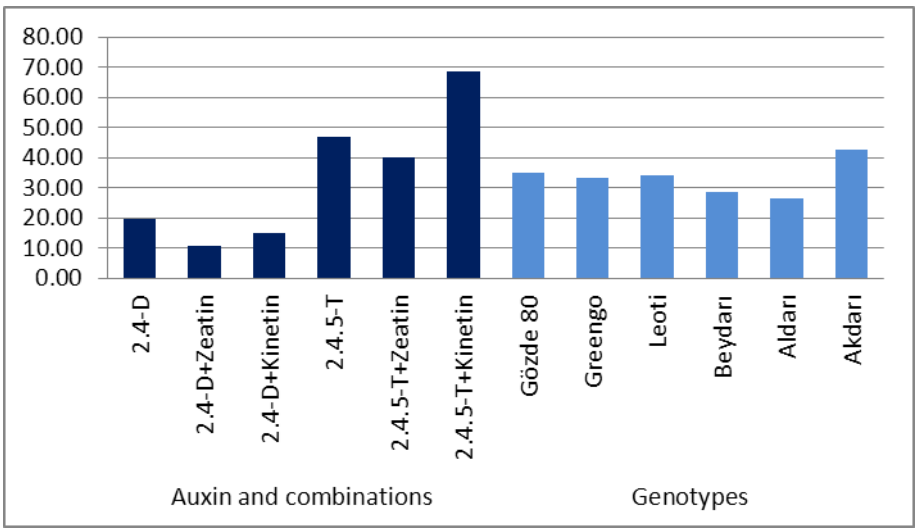

Figure 2. *The effects of sorghum cultivars and different auxin types and their combinations on the rooting percentage (%)*

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The highest rooting percentage was obtained from Akdarı cultivar similar as callus induction percentage. However, silage sorghum cultivars like Greengo giving the highest shooting showed values close to Akdarı. 2,4,5-T and combinations were found to be more effective than 2,4-D and combinations in rooting and subsequent development. The addition of kinetin with the 2,4,5-T to the callus induction medium affected shoot and rooting formation positively despite phenolic compound formation. Simpson et al. (1982) reported that kinetin positively affected the uptake of NO_3 in roots and increased nitrogen content in roots and shoots of wheat. Gupta et al. (2006) stated that kinetin-like cytokinins should be added to the callus formation medium to overcome the genotypic limitations of plant regeneration.

Conclusion

An efficient regeneration system was developed by using mature embryos in sorghum. Akdarı (Tannin-free cultivar) showed the high callus induction and rooting percentages. However, silage cultivars seem to be better in terms of shoot formation and no difference with Akdarı in regard to rooting. While 2,4-D gave better callus induction, 2,4,5-T showed better values of the shoot and rooting formation. The formation of phenolic compounds affected plant regeneration negatively in all cultures. In this study, an efficient regeneration protocol despite the formation of phenolic compounds was developed from mature embryos, an unlimited explant source for tissue culture and gene transfer studies. It was concluded that 1 mg/l 2,4,5-T combined with 0.5 mg/l kinetin should be advised for prolific callus induction and regeneration obtained from somatic embryogenesis by using mature embryos in sorghum.

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