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In vitro Plant Regeneration Efficiency from Different Explants of Local Sainfoin Ecotype (*Onobrychis sativa*)

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ABSTRACT

Local populations and ecotypes are important genetic resources with genetic diversity. It is possible to protect threatened genotypes and increase biodiversity with *in vitro* tissue culture techniques. Explant type is of great importance among the factors affecting the success of *in vitro* regeneration and callus induction. In this study, Sainfoin seeds belonging to the Gözlü ecotype were used as experiment material. MS media were prepared with TDZ and combinations of IBA-NAA. Callus induction and shoot regeneration rates of hypocotyl, leaf and cotyledon explants were determined in the sainfoin ecotype. According to the results of the study, the best callus formation and shoot regeneration were obtained from the hypocotyl explant in an MS medium containing 2 mg/L TDZ. In the present study, important results were obtained that will contribute to the regeneration studies of local ecotypes and wild species, genetic transformation and increasing genetic diversity.

Keywords**:** *In vitro* regeneration, callus induction, regeneration efficiency, sainfoin ecotype

Introduction

Sainfoin (*Onobrychis sativa*) is an important forage crop used as animal feed all over the world. Anatolia-Iran-Caucasus regions constitute the gene centers of the sainfoin. Turkey is the natural habitat of the sainfoin. 52 sainfoin species are distributed in Turkey and more than half of these sainfoin species are endemic (Avcı 2010; Beyaz 2019).

Turkey is one of the gene centers of sainfoin, as in many other plant species (Harlan, 1951). Local populations and ecotypes are important genetic resource with genetic diversity. Local plant populations and wild species are of great agricultural importance due to their high stress tolerance and environmental adaptation capability (Kishii 2019; Akman and Karaduman 2021). Local ecotypes are the target of breeding studies and biotechnological approaches. Ecotypes that are genotypically adapted to certain environmental conditions have high genetic diversity within their species (Turesson, 1922). Wide genetic diversity of ecotypes is of great importance in breeding programs (Avcı et al. 2014).

In vitro propagation of local populations and genetic resources is successful and reproducible methods to preserve the germplasm of species (Ergül et al. 2018; Deb et al. 2019). In addition, tissue culture methods can be used successfully to increase genetic variation or manipulations on plant material. Successful *in vitro* regeneration protocol has been used in many fields such as somaclonal variation, *in vitro* mutation, tolerance to biotic and abiotic stress, diploid plant production, production of secondary metabolites, production of economically important transgenic plants, CRISPR genetic editing and development of somatic hybrids (Oğuz et al. 2021).

Direct or indirect plant regeneration from plant tissues and parts is possible with somatic embryogenesis and organogenesis. Callus induction, somatic embryogenesis and plant regeneration have been performed by many researchers with different *in vitro* techniques and plant growth regulators (PGR) combinations (Özcan et al. 1996; Sancak 1999; Kamalvand and Karamian 2013; Beyaz 2014; Yildiz and

Ekiz 2014; Honarmand et al. 2016; Beyaz 2019, Uzun and Yükselgüngör 2020; Uysal and Topbaş 2021). The success of *in vitro* regeneration affect by genotype, explant type, the composition of the MS medium, and PGR.

On the other hand, studies on *in vitro* regeneration of endemic and local *Onobrychis* species are very limited (Uzun and Yükselgüngör 2020). In this study, *in vitro* regeneration efficiency from different explants of the local sainfoin ecotype was determined.

Materials and Methods

In this study, Gözlü sainfoin (*Onobrychis sativa*) ecotype were used as sources of plant explant. Sainfoin seeds were sterilized with 100%, 90%, 80%, 70% and 60% commercial bleach. In all sterilization experiments, after a 15-minute treatment period, rinsing with sterile water 3 times for 5 minutes. In the experiments, a medium (MS0) containing MS mineral salts and vitamins (Murashige and Skoog 1962) and 3% sucrose, 5g/L agar (Type A, Sigma). The planted seeds in MS media were grow up at 24°C for 3 weeks in a photoperiod of 16 hours light and 8 hours dark. Cotyledon, hypocotyl and leaf explants were isolated three weeks old sainfoin plantlets.

For the callus and regeneration experiment, *in vitro* media were prepared with combinations of 0.5, 1, 2 mg/L TDZ, NAA and BAP. As Sancak (1999) stated that the rooting media was added 1 mg/L IBA. Rooted regenerants were transferred to pots with peat and soil at a ratio of 1:1. Acclimatization was carried out in the plant growth chamber under 50% constant humidity, plants with covering a transparent plastic bag.

All regeneration experiments were established according to the random plot design with three replications, and statistical analyzes were performed using the Tukey test in the "IBM SPSS 22 software" program.

Results

The results obtained from sterilization experiments using different percentage of commercial bleach are given in Table 1. Successful results were obtained in the sterilization of hulled seeds with a high rate of bleach. However, it caused a decrease in germination rate (Table 1). On the other hand, the germination rate of hulled seeds was higher at low bleach concentrations, but the contamination rate was not decreased. In addition, *in vitro* seedling growth of these seeds was deficient.

The sterilization of dehulled seeds, contamination rates was zero in 100%, 90% and 80% bleach, but germination rates were determined as 81%, 79% and 76%, respectively. The lowest contamination rate and the best germination rate were determined in the dehulled seeds with 70% bleach. The germination rate of seeds sterilized with 70% bleach was 98% (Table 1).

Effect of TDZ on callus formation and shoot regeneration

Callus induction and shoot regeneration results of sainfoin hypocotyl, leaf and cotyledon explants from MS media containing different rate of TDZ are given in Table 2. High callus formation was determined for the hypocotyl explant in MS medium containing 1 and 2 mg/L TDZ. On the other hand, the statistical difference between the callus rates of the hypocotyl explant obtained in 0.5, 1 and 2 mg/L TDZ was insignificant ($p>0.01$). The highest callus ratio from leaf explant was obtained at 0.5 mg/L TDZ ($p<0.01$). The difference between the callus rates obtained from the medium containing 1 and 2 mg/L TDZ was insignificant ($p>0.01$) (Table 2). Although there is an increase in the callus rates obtained from the cotyledon explant compared to the control ($p<0.01$), the difference between TDZ treatments are insignificant ($p>0.01$).

The highest average shoot number obtained from the hypocotyl explant was measured in MS medium containing 2 mg/L TDZ (10.76). The highest average number of shoots in the leaf explant was determined in the media containing 1 and 2 mg/L TDZ (2.40 and 2.73 respectively) (Table 2). In the cotyledon explant, the highest shoot number (6.26) was obtained from MS medium containing 2 mg/L TDZ ($p<0.01$) (Figure 1).

The effect of TDZ and BAP combinations on callus formation and shoot regeneration

In the study, hypocotyl, leaf and cotyledon explants were cultured in MS media containing a combination of TDZ and BAP. All explants were observed during 1-12 weeks after they were placed in the culture medium. Callus formations were determined 2-8 weeks after the explants transferred to the culture medium. The highest callus induction rate in hypocotyl and cotyledon explants was determined in MS media containing 1 mg/L TDZ + 0.5 mg/L BAP (respectively 83.33% and 23.30%) (Table 3). Compared to other TDZ+BAP combinations in the experiment, 1 mg/L TDZ + 0.5 mg/L BAP was found to be statistically significant ($p<0.01$).

The highest callus formation in the leaf explant was determined in MS containing 1 mg/L TDZ +1 mg/L BAP and 2 mg/L TDZ + 0.5 mg/L BAP (33.30% and 30.00%, respectively). However, the difference between these treatments are statistically insignificant ($p>0.01$). No shoot formation occurred from the cotyledon explant in MS media containing TDZ and BAP combinations. The highest average shoot number of hypocotyl explant was obtained in the nutrient medium containing 1 TDZ mg/L + 0.5 BAP mg/L ($p<0.01$) (Table 3). On the other hand, shoots were obtained from the leaf explant in medium containing only 0.5 mg/L TDZ + 1 mg/L BAP (2.60) (Table 3).

The effect of TDZ and NAA combinations on callus formation and shoot regeneration

Callus and shoot regenerations in MS media containing TDZ and NAA combinations are given in Table 3. The highest callus formation in the hypocotyl explant was determined in the medium containing 0.5 mg/L TDZ + 2 mg/L NAA. The best callus formation in leaf explant was obtained in 0.5 and 1 mg/L TDZ + NAA combinations. The difference between 0.5 and 1 mg/L TDZ + NAA combinations was statistically insignificant ($p>0.01$). Callus induction obtained from leaf explant at 2 mg/L TDS doses were less than 0.5 and 1 TDZ + NAA doses ($p<0.01$). In the cotyledon explant, the highest callus formation was obtained from MS medium containing 1 mg/L TDZ + 2 NAA mg/L (Table 4).

The highest average shoot number from the hypocotyl explant was obtained from MS medium containing 2 mg/L TDZ + 0.5 mg/L NAA (6.33). It was observed that increasing NAA negatively affected shoot formation (Table 4). In the leaf explant, the highest average shoot number was obtained from MS medium containing 2 mg/L TDZ + 1 mg/L NAA. In the experiment, shoot regeneration did not occur in the cotyledon explant (Table 4). The shoots obtained from leaf and hypocotyl explants were transferred to plastic pots after rooting in MS medium containing 1 mg/L IBA (Figure 2). All transplanted plants into the soil continued to grow and develop after acclimatization.

Discussion

Somatic embryogenesis is defined as the formation of embryos from vegetative cells *in vitro* (Hatipoğlu 2012). Developing genetic variability in existing varieties with somatic embryogenesis and *in vitro* techniques is routinely applied in the production of species that are in danger of extinction and in the production of species that are difficult to reproduce (Erişen, 2005).

Obtaining sterile explants is one of the major factors affecting success in tissue culture techniques. Contamination from plant material has to be eliminated with successful sterilization protocols. In this study, sterile plants were obtained with 70% commercial bleach. In particular, sterilization of dehulled seed was more successful. Ergül et al. (2018) reported that sterile plants were obtained by removing the seed coat with chemical and physical interventions for the sterilization of seeds of wild Beta germplasm. In this study, the seed coat was removed by physical interventions. In this way, contamination in the deep surfaces of the seed was eliminated.

The content of auxin and cytokines contained *in vitro* tissue culture media is of great importance for callus and regeneration success. Inducing hormones are needed in the growth media for the regeneration of explants isolated from the plant. Although the inducer required for organogenesis is removed from media after a certain period of time, the growth and development continue (Ikeuchı et al. 2013). On the other hand, a callus may occur in the presence of high auxin or equal auxin-cytokine hormone in the MS medium (Mohnen 1994). Besides, Oğuz et al. (2021) reported that the endogenous hormone content of plant tissues has a significant effect on *in vitro* regeneration and callus formation. In our study, callus induction and shoot regeneration were obtained from different explant types with the effect of IBA and NAA added to TDZ-containing media. In addition, it is thought that callus formation in MS medium with high TDZ is caused by the endogenous auxin level of the plant explant.

Frequency of regeneration and callus formation are hereditary characters affected by genotypes (Kagami et al. 2016). The differences between the results obtained in sainfoin *in vitro* regeneration and callus induction studies and our study are due to genotypic differences. The rate of shoot regeneration is low in MS media containing TDZ + BAP and TDZ + NAA compared to media containing only TDZ in all explants used in the study. The highest shoot regeneration was obtained from the hypocotyl explant in nutrient media containing 2 mg/L TDZ. Garshasbi et al. (2012) reported that more than 60% of shoots rooted successfully in four weeks in semi-strength MS medium supplemented with 1 mg/L IBA. In the experiment, MS media containing 1mg/L IBA were used for rooting media (Figure 1). Root formation was successfully achieved in approximately 80% of the regenerants. Besides, the fact that all plants transferred to the soil continue to grow after acclimatization is closely related to the high efficiency of *in vitro* rooting.

Conclusions

Tissue culture techniques have great importance in many areas such as the manipulation of cell tissues, biomaterial production, and elucidation of the mechanisms underlying plant growth processes. Besides, it is possible to increase and preserve the genetic diversity to be used in breeding studies with *in vitro* regeneration and callus formation of local populations and ecotypes. In this study, high degree of callus and shoot regeneration was obtained from MS media containing 2 mg/L TDZ. In addition, important results were obtained for the *in vitro* propagation and conservation of local sainfoin ecotypes. It is clear that these results could contributions to other *in vitro* based techniques such as tissue culture, genetic transformation or new biodiversity studies.

Table 1. Effect of different bleach concentrations on sainfoin seed sterilization and germination rate.

Bleach (%)	Hulled seed		Dehulled Seed	
	Contamination Rate (%)	Germination Rate (%)	Contamination Rate (%)	Germination Rate (%)
100	0	89[b]	0	81[b]
90	0	96[a]	0	79[b]
80	14	95[a]	0	76[b]
70	20	88[b]	0	98[a]
60	26	83[b]	11.00	94[a]

* The difference between the different letters in the same column is significant at the 0.01 level (p<0.01)

Table 2. The effect of TDZ on callus and shoot formation from hypocotyl, leaf and cotyledon explants in sainfoin.

Plant Growth Regulators (mg/L)	Callus Induction (%)			Average Number of Shoots		
TDZ	Hypocotyl	Leaf	Cotyledon	Hypocotyl	Leaf	Cotyledon
0.0	55.00[b]	43.30[c]	60.00[c]	1.36[c]	1.10[b]	1.22[d]
0.5	96.66[a]	96.66[a]	96.66[a]	6.80[b]	1.43[b]	3.10[c]
1.0	100.00[a]	90.00[a]	96.66[a]	8.80[a]	2.40[a]	4.66[b]
2.0	100.00[a]	93.30[a]	96.66[a]	10.76[a]	2.73[a]	6.26[a]

* The difference between the different letters in the same column is significant at the 0.01 level (p<0.01)

Table 3. The effect of TDZ+BAP combination on callus and shoot formation from hypocotyl, leaf and cotyledon explants in sainfoin.

Plant Growth Regulators (mg/L)		Callus Induction (%)			Average Number of Shoots		
TDZ	BAP	Hypocotyl	Leaf	Cotyledon	Hypocotyl	Leaf	Cotyledon
0.5	0.5	16.00[c]	3.30[d]	0.00[d]	0.00[d]	0.00[b]	0.00
0.5	1.0	3.30[d]	3.30[d]	0.00[d]	2.60[b]	2.60[a]	0.00
0.5	2.0	0.00[f]	6.60[c]	0.00[d]	0.00[d]	0.00[b]	0.00
1.0	0.5	83.33[a]	16.60[b]	23.30[a]	2.86[a]	0.00[b]	0.00
1.0	1.0	30.00[b]	33.30[a]	3.30[c]	1.30[c]	0.00[b]	0.00
1.0	2.0	3.30[d]	10.00[c]	10.00[b]	0.00[d]	0.00[b]	0.00
2.0	0.5	0.00[f]	30.00[a]	0.00[d]	0.00[d]	0.00[b]	0.00
2.0	1.0	0.00[f]	16.60[b]	0.00[d]	0.00[d]	0.00[b]	0.00
2.0	2.0	3.30[d]	3.30[d]	0.00[d]	0.00[d]	0.00[b]	0.00

* The difference between the different letters in the same column is significant at the 0.01 level (p<0.01)

Table 4. The effect of TDZ and NAA combinations on callus formation and shoot regeneration.

Plant Growth Regulators (mg/L)		Callus Induction (%)			Average Number of Shoots		
TDZ	**NAA**	**Hypocotyl**	**Leaf**	**Cotyledon**	**Hypocotyl**	**Leaf**	**Cotyledon**
0.5	0.5	93.30[a]	86.60[a]	0.30[e]	0.00[c]	0.00[c]	0.00
0.5	1.0	93.30[a]	93.30[a]	53.30[b]	0.00[c]	0.00[c]	0.00
0.5	2.0	100.00[a]	96.60[a]	43.30[b]	0.00[c]	0.00[c]	0.00
1.0	0.5	50.00[b]	90.00[a]	3.30[e]	0.00[c]	0.00[c]	0.00
1.0	1.0	36.60[c]	96.60[a]	3.30[e]	0.00[c]	0.00[c]	0.00
1.0	2.0	63.30[b]	96.60[a]	63.30[a]	0.00[c]	0.00[c]	0.00
2.0	0.5	90.00[a]	50.00[c]	16.60[c]	6.30[a]	0.00[c]	0.00
2.0	1.0	96.60[a]	73.30[b]	76.60[a]	4.60[a]	4.00[a]	0.00
2.0	2.0	63.30[b]	63.30[b]	6.60[d]	2.00[b]	1.10[b]	0.00

* The difference between the different letters in the same column is significant at the 0.01 level ($p<0.01$)

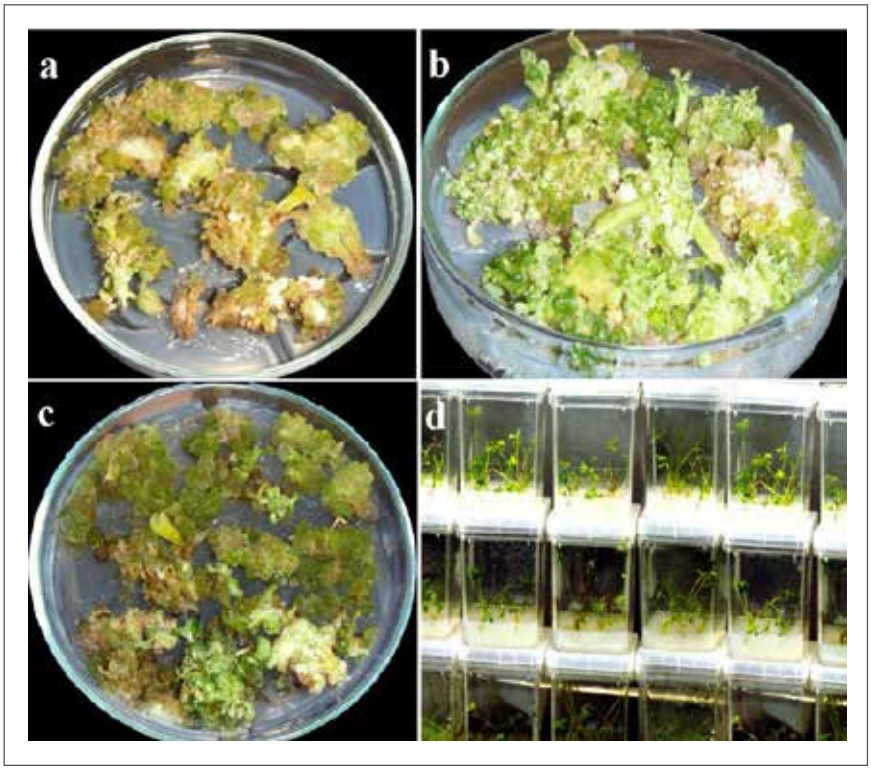

Figure 1. *In vitro* plant regeneration of sainfoin from different explants (Original)
a) Callus formation and shoot regeneration in leaf explant in MS medium containing 2.0 mg/L TDZ,
b) Callus formation and shoot regeneration from hypocotyl explant,
c) Callus formation and shoot regeneration at the end of 6 weeks from the cotyledon explant
d) Development of plantlets in rooting medium

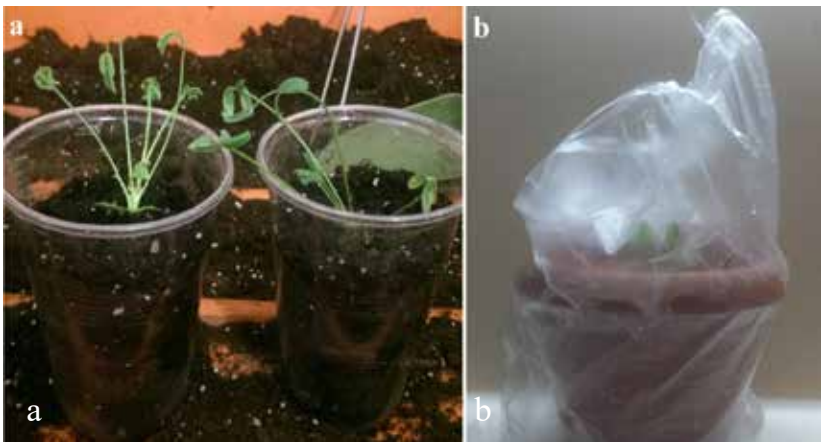

Figure 2. Acclimatization stage of *in vitro* plantlets (Original)
a) Transfer of the plant to the soil after root development,
b) Plants covered with a transparent plastic bag for acclimatization

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