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SOMATIC EMBRYOGENESIS IN SAINFOIN (*Onobrychis viciifolia Scop.*)

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Abstract
Sainfoin (Onobrychis viciifolia Scop.) is a perennial legume used for forage production. The aim of this study was to investigate the effects of different basal media (Murashige&Skoog-MS, Schenk& Hildebrant-SH, Gamborg-B5 or CHU-N6), explant types (cotyledon and hypocotyl), and growth regulators (kinetin and thidiazuron) on the induction of somatic embryogenesis in sainfoin. For this purpose, hypocotyl and cotyledon explants isolated from in vitro grown sterile seedlings were cultured in solid MS, SH, B5, or N6 basal media supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.2 mg/L kinetin or thidiazuron, and 1 g/L proline. The developing embryonic callus was then transferred to a solid MS medium containing 3% sucrose. As a result of the experiment, callus weights per explant varied between 248.3-534.7 mg, 408.7-609.1, 220.3-537 mg and 127.3-428.3 mg in MS, B5, N6, and SH basal media respectively, depending on explants and growth regulators. Average callus weights were higher in B5 and N6 basal media than in MS and SH media, TDZ-containing media than kinetin containing media and cotyledon explant than hypocotyl explant. The highest frequency of explants developing somatic embryos (mean 33.3%) was obtained from kinetin-containing MS or B5 basal media, while the highest embryo per explant (mean 2.6 somatic embryo per explant) was obtained from kinetin-containing MS medium. The somatic embryo number per hypocotyl explant was higher than that of cotyledon explants.

Keywords: *Onobrychis viciifolia, basal medium, growth regulators, explant, somatic embryogenesis.*

1. INTRODUCTION

Sainfoin (*Onobrychis viciifolia* Scop. = *Onobrychis sativa* Lam.) is a perennial forage crops within the *Onobrychis* genus in the Fabaceae family, which has been cultivated for hundreds of years in many parts of the world (Carbonero et al., 2011). Sainfoin is widely distributed in the northern temperate regions of the world, and the Eastern Mediterranean and Western Asia, especially Iran and Turkey are considered the center of diversity of this species (Yildiz et al., 1999; Bhattarai et al., 2016).
Sainfoin is quite resistant to cold and drought. It gives good yields where other forage crops fail due to drought (Açıkgöz, 2021). After the seedling period, it is particularly resistant to cold. Sainfoin can grow in calcareous and in very low-yield soils (Tan and Sancak, 2009). It is of great importance in terms of soil improvement due to its strong root development and being a legume plant. It is one of the ideal plants that can be used on sloping lands in terms of erosion control (Tan and Sancak, 2009; Yüksek and Yüksek, 2015). It is more productive than alfalfa in unirrigated arid areas, but it cannot compete with alfalfa in irrigated areas, It can grow in barren, weak, gravelly and calcareous soils where alfalfa cannot grow (Tan and Sancak, 2009; Açıkgöz, 2021). It has high nutritional

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value and is rich in nitrogen-free essential substances, crude oil and crude protein (Açıkgöz, 2021). In addition, condensed tannins (proanthocyanidins) found in sainfoin reduce parasites in the digestive system of ruminant animals and provide environmental benefits by reducing methane emissions from ruminant animals (Bhattarai et al., 2018). Unlike alfalfa, sainfoin does not cause bloat in grazing animals (Açıkgöz, 2021). Sainfoin is also resistant to *Hypera postica*, which causes significant damage to alfalfa (Bhattarai et al., 2016). However, in sainfoin cultivation, *Sphenoptera carceli* and *Bembecia scopigera* cause economic damage in Turkey, and these pests are encountered in all areas where sainfoin cultivation is carried out (Açıkgöz, 2021). Studies have been carried out to develop resistant or tolerant varieties against these pests, but it has been determined that the examined species or varieties are not resistant to pests (Büyükburç et al., 1991; Açıkgöz, 2021).

Somatic embryogenesis is defined as the process of development of bipolar-structured, zygotic embryo-like structures from cells that are non-zygotic and have no connection with the original tissue (Sevindik et al., 2021). Somatic embryogenesis is widely used in studies such as rapid propagation of new and elite genotypes, synthetic seed production, in vitro selection approaches for various biotic and abiotic stress factors, and gene transfer (Kamle et al., 2011). There are many factors that affect somatic embryogenesis. These factors include genotype, explant source, medium, growth regulators, and external environmental factors, including physical culture conditions such as light and temperature (Gökdoğan, 2013). The aim of this study was to investigate the effects of different basal media, explant types, and growth regulators on somatic embryogenesis in sainfoin.

2. MATERIALS AND METHODS

The research was carried out in the Tissue Culture Laboratory of Erciyes University Faculty of Agriculture, Department of Field Crops. (*Onobrychis viciifolia*) ecotype (Gözlü) were used in the experiments. The peeled sainfoin seeds were mixed with a magnetic stirrer for 15 minutes in 50% bleach (ACE) for and then rinsed 3 times with sterile pure water. Sterilized seeds were placed in MS medium containing 3% sucrose and solidified with 0.7% agar. Hypocotyls and cotyledons isolated from 10-15 days old sterile seedlings grown in vitro were cultured on solid MS, SH, B5 or N6 basal medium containing 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/L kinetin or thidiazuron (TDZ) and 1 g/L proline (Table 1.) Developing embryonic calli were then transferred to MS medium solidified with 0.7% agar containing 3% sucrose.

Pure water was used to prepare the nutrient media. pH of the media was adjusted to 5.6-5.8 using 1 N NaOH or HCl. Sterilization of the media and glassware was carried out using an autoclave (under 1.5 atmospheres of pressure, at 121 °C for 20 minutes). All cultures were grown in a 16-hour light and 8-hour dark photoperiod at 24±2 ˚C and 5000 lux light intensity.

In the experiment, 2 different explants (hypocotyl and cotyledon), 2 diffrent growth regulators (Kinetin and TDZ) and 4 diferent basal media (MS, SH, B5 and N6) were used. The experiment was conducted with 3 replications and 6 explants in each replication. The results obtained in the study were subjected to analysis of variance using SPSS, and the differences between the means were determined with the Duncan’s multiple range test.

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Table 1. Basal media, growth regulators and explants used for somatic embryogenesis

Treatments		
Growth regulators	**Explants**	**Basal media**
0.2 mg/L kinetin	Cotyledon	MS (Duchefa, M0222)
		B5 (Duchefa, G0210)
		N6 (Duchefa, C0204)
		SH (Duchefa, S225; S0411)
	Hypocotyl	MS
		B5
		N6
		SH
0.2 mg/L TDZ	Cotyledon	MS
		B5
		N6
		SH
	Hypocotyl	MS
		B5
		N6
		SH
2 mg/L 2,4-D, 1 g/L proline, 3% sucrose were added to all media, adjusted to pH=5.6-5.8 and solidified with 0.7% agar.		

3. RESULTS AND DISCUSSIONS

As a result of variance analysis of callus weights; the explants, growth regulators and basal media were found to be significant at the 1% level. Callus weights per explant varied between 248.3-534.7 mg, 408.7-609.1, 220.3-537 mg and 127.3-428.3 mg in MS, B5, N6, and SH basal media respectively, depending on explants and growth regulators (Table 2). Average callus weights were higher in B5 and N6 basal media than in MS and SH media, TDZ-containing media than kinetin containing media and cotyledon explant than hypocotyl explant.

Table 2. Callus weights (mg) per explant obtained from hypocotyl and cotyledon explants of sainfoin in different growth regulators and basal media

Treatments		**Basal media**				**Means**
Growth regulators	**Explants**	**MS**	**B5**	**N6**	**SH**	
Kinetin	Cotyledon	345.3	525.7	477.0	303.0	412.8
	Hypocotyl	248.3	408.7	220.3	127.3	251.2
TDZ	Cotyledon	534.7	568.3	536.0	428.3	516.8
	Hypocotyl	297.0	609.1	537.0	145.3	397.1
Means		**356.3 bc^1**	**527.9 a**	**442.6 ab**	**251.0 c**	**Means**
Growth regulators	Kinetin	*296.8*	*467.2*	*348.7*	*215.2*	***332.0 b^2***
	TDZ	*415.8*	*588.7*	*536.5*	*286.8*	***457.0 a***
Explants	Cotyledon	440.0	547.0	506.5	365.7	**464.8 a^2**
	Hypocotyl	272.7	508.9	378.7	136.3	**324.1 b**

The difference between the means shown in different lowercase letters in the same row (^{1}) and columns (^{2}) is significant at the 0.05 level

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As a result of variance analysis of the frequency of explants developing somatic embryos and mean number of somatic embryos per explant; the explants, growth regulators, basal media and the interaction growth regulators × basal media were found to be significant at the 1% level. The frequency of explants developing somatic embryos varied between 0-38.9%, 11.1-44.4%, 0-22.2 % and 11.1-22.2% in MS, B5, N6, and SH basal media respectively, depending on explants and growth regulators (Table 3). The highest frequency of explants developing somatic embryos (mean 33.3%) was obtained from kinetin-containing MS or B5 basal media. Somatic embryos did not occur in MS and N6 nutrient media containing TDZ. The number of somatic embryos per explant varied between 0-3.5, and the highest value was obtained from the hypocotyl explant on MS basal medium containing kinetin (Table 4).

Table 3. The frequency of hypocotyl and cotyledon explants developing somatic embryo of sainfoin in different growth regulators and basal media (%)

Treatments		**Basal media**				**Means**
Growth regulators	**Explants**	**MS**	**B5**	**N6**	**SH**	
Kinetin	Cotyledon	27.8	22.2	0	11.1	15.3
	Hypocotyl	38.9	44.4	22.2	16.7	30.6
TDZ	Cotyledon	0	11.1	0	11.1	5.6
	Hypocotyl	0	22.2	0	22.2	11.1
Means		**16.7 a[1]**	**25.0 a**	**5.6 b**	**15.2 a**	**Means**
Growth regulators	Kinetin	*33.3 a[3]*	*33.3 a*	*11.1 bc*	*13.9 bc*	**22.9 a[2]**
	TDZ	*0 c*	*16.7 b*	*0 c*	*16.7 b*	**8.3 b**
Explants	Cotyledon	13.9	16.7	0	11.1	**10.4 b[2]**
	Hypocotyl	19.5	33.3	11.1	19.5	**20.8 a**

The difference between the means shown in different lowercase letters in the same row (1), columns (2) and raw/columns (3) is significant at the 0.05 level

Table 4. Mean number of somatic embryos per explant obtained from hypocotyl and cotyledon explants of sainfoin in different growth regulators and basal media

Treatments		**Basal media**				**Means**
Growth regulators	**Explants**	**MS**	**B5**	**N6**	**SH**	
Kinetin	Cotyledon	1.7	0.9	0	0.6	0.8
	Hypocotyl	3.5	2.3	1.2	0.6	1.9
TDZ	Cotyledon	0	0.4	0	0.3	0.2
	Hypocotyl	0	1.3	0	0.6	0.5
Ortalamalar		**1.3 a[1]**	**1.2 a**	**0.3 b**	**0.5 b**	**Means**
Growth regulators	Kinetin	*2.6 a[3]*	*1.6 b*	*0.6 c*	*0.6 c*	**1.3 a[2]**
	TDZ	*0 c*	*0.9 bc*	*0 c*	*0.4 c*	**0.3 b**
Explants	Cotyledon	0.8	0.7	0	0.4	**0.5 b[2]**
	Hypocotyl	1.7	1.8	0.6	0.6	**1.2 a**

The difference between the means shown in different lowercase letters in the same row (1), columns (2) and raw/columns (3) is significant at the 0.05 level
Numbers of somatic embryos per explant=total number of somatic embryo per petri dishes/total number of explant per petri dishes

In general, the effect of kinetin on somatic embryo formation was higher than TDZ in this study. Similarly, Niazian et al. (2017) also determined higher somatic embryo formation in 15-day-old hypocotyl explants (28.3 embryos) cultured in MS medium containing 1.5 mg/L 2,4-D and 0.5 mg/L kinetin in *Carum copticum*. Bhusare et al. (2020) reported that the most effective medium in

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embryonic callus and indirect somatic embryo induction (95.5 and 9.8% somatic embryo/explant) was kinetin containing media in *Digitalis lanata*. Mendez-Hernandez et al. (2019) and Bhusare et al. (2020) reported that the type of auxin or cytokinin or their combinations and appropriate concentrations vary from species to species and need to be studied to obtain the best media for the induction of somatic embryos. As a matter of fact, in studies conducted in different species, it has been reported by different researchers that TDZ is very effective on somatic embryogenesis (Verma et al., 2012; Çölgeçen, 2017; Verma et al., 2022).
One of the factors affecting success in tissue culture is the basal nutrienth medium (Kodaz et al. 2021). When the frequency of explants developing somatic embryos and mean number of somatic embryos per explant were examined, the highest values were obtained from MS and B5 basal media. Pinto et al. (2008) stated that MS and B5 are the best basal media for somatic embryo induction and regeneration in *Eucalyptus globulus*, and in general, MS is the best basal nutrient medium for expression, regardless of the medium used during induction. Abbasi et al. (2016) determined the highest somatic embryo formation and proliferation by subculturing embryonic calli in B5 medium containing 0.5 mg/L 2,4-D and 1.5 g/L TDZ in *Silybum marianum*. It has also been reported that MS0 medium is more sensitive for the growth and maturation of somatic embryos. Sangra et al. (2019) reported that while the highest embryo yield and plant transformation rate were in the B5H-B5 system (callus development-embryo development), the highest embryonic sustainability was in the SH4K-BOi2Y system in *Medicago sativa*. As stated in the studies summarized above, differences may occur among basal nutrient media in terms of somatic embryo induction and development. The hypocotyl explant exhibited the highest values in terms of somatic embryo formation frequency and the number of somatic embryos per explant. In previous studies conducted on sainfoin, successful results were obtained from the hypocotyl explant in terms of shoot regeneration (Turgut et al., 2022; Uzun, 2012).

4. CONCLUSIONS

This paper reports a procedure for indirect somatic embryogenesis through embryogenic callus formation in sainfoin. The highest frequency of explants developing somatic embryos (mean 33.3%) was obtained from kinetin-containing MS or B5 basal media, while the highest somatic embryo per explant (mean 2.6 somatic embryo per explant) was obtained from kinetin-containing MS medium. The frequency of somatic embriyo formation and the somatic embryo number in hypocotyl was higher than that of cotyledon explants. The procedure described here can be improved in efficiency with future studies.

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