Caucasian Journal of Science

Volume: 6, Issue: 2, Year : 2019 www.cjoscience.com e-ISSN: 2148-6840

The Investigation of In Vitro Callus Formation, Regeneration and Micropropagation of Devegülü Hollyhock, a Medicinal Plant

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Araştırma Makalesi Research Article	Moleküler Biyoloji ve Genetik Molecular Biology and Genetics	Geliş Tarihi/Received 05.11.2019	Kabul Tarihi/Accepted 07.12.2019

Abstract: Devegülü hollyhock is used by public for the treatment of certain diseases (such as tonsillitis, stomach ulcer, duodenal ulcer, pneumonia, urinary tract infections and alopecia). This study investigated the appropriate protocols for *in vitro* callus formation, regeneration and micropropagation of various explants of 14-day and 28-day old Devegülü seedlings germinated from the seeds in a sterile environment. Different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) were used as plant growth regulator and Murashige and Skoog basal medium was used as basic medium. Regenerated plantlets were firstly transferred to rooting medium to promote root formation, after that they were planted into soil. Both of 14-day and 28-day explants produced a higher rate of callus in MS medium including 2,4-D auxin hormone. The regenerated plantlets were obtained from the shoot tip and node explants of 28 days old seedlings after incubation in MS medium for 6 weeks. Direct regeneration from the node and shoot tip was observed in this hormone free medium. Micropropagation rates were 44.4 % and 12.5 % for node and shoot tip explants, respectively. With this study, callus formation, regeneration and micropropagation of the shoot tip and nodes was successfully carried for the first time.

Keywords: Devegülü, *In Vitro* Regeneration, Callus, Hollyhock.

1. INTRODUCTION

Alcea kurdica (Schlecht) Alef. is called Devegülü in Turkey (Güner et al., 2012). It is a perennial herbaceous plant belonging to the genus *Alcea*, called hollyhocks, known for its colourful and splendid flowers belonging to the Malvacea family with its 40 subspecies (Uzunhisarcıklı and Vural, 2012). It is one of the hollyhock species distributed in east of Turkey, north of Iraq, Northwest of Iran at 1750-2500 meters altitude (Uzunhisarcıklı and Vural, 2012). Cowan (1999) reported from Stockwell (1988) and Thomson (1978) that hollyhocks were used by the extinct Neandarthal people who are believed to have survived in

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Iraq some 60000 years ago, and today they are still used for medicinal purposes. Devegülü plant is used by native population in the treatment of various diseases such as tonsillitis, stomach ulcer, duodenal ulcer, pneumonia, urinary tract infections and allopecia due to its antioxidant (Bouayed et al., 2007) and antimicrobial (Qader and Awad, 2014) properties. Görmez et al., (2019) reported that different vegetation stages affected Devegülü plant's macro and microelement content and protein content. Secondary metabolites isolated from Alcea species showed to have no such non-toxic effects as psychoactive and mind-blurring because of low contents of alkaloids, but on the contrary, have antioxidant, antimicrobial, antiviral and liver protective properties on humans and other mammals (Azab, 2017).

Medicinal plants cannot be obtained from the natural environment when needed and in the desired amounts. Plant tissue culture is a method to produce new plants from different explant types (leaf, cotyledon, hypocotyl, root, stem, node and shoot tip etc.) using artificial media including macro and micro-elements for plant growth, plant growth regulators to promote cell division and growth under sterile conditions and controlled environment (Mansuroglu and Gurel, 2001). Continuous and high-quality micropropagation of medicinal plants in tissue culture prevents the extinction and habitat destruction of plants and also keeps them away from the remnants of pesticides that threaten animal and environmental health (Murch et al., 2000).

Although numerous tissue culture and regeneration studies have been conducted with members of the Malvacea family such as *Gossypium hirsitum* L., (Özyiğit et al., 2007), *Hibiscus sabdariffa* (Raoul et al., 2010), *Alcea rosea* (Mubashrah et al., 2012) and *Hibiscus cannabinus* L. (Samanthi et al., 2013), studies on tissue culture of Devegülü (*Alcea kurdica* (Schlecht) Alef.) have not been reported yet.

The aim of this study was to investigate *in vitro* callus formation, regeneration and micropropagation of Devegülü hollyhock which was used as a medicinal plant among public.

2. MATERIALS AND METHODS

2.1. Plant Material

Seeds of Devegülü were collected in August and September 2017 from Van region. Impurities were removed from the seeds. Later they were laid on drying papers. Dried seeds were packaged and stored at +4°C.

2.2. Regeneration of the Devegülü Hollyhock

Murashige & Skoog (MS) medium (4.40g L^{-1}) was used in tissue culture studies. Sucrose (30 g L^{-1}) as the carbohydrate source and plant agar as the gelling agent were added before autoclaving (7g L^{-1}). The pH of the medium was adjusted to 5.7-5.8 using 1M sodium hydroxide (NaOH) and 1M hydrochloric acid (HCl). The medium was sterilized by keeping under a pressure of 1.5 atmospheres at 121ºC for 20 minutes.

Stock solutions of plant growth regulators (PGR) at 1 mg mL^{-1} concentrations were prepared and added to nutrient media and poured into 90x15 mm sterile petri dishes. Different concentrations of 2,4- dichlorophenoxy acetic acid were used as plant growth regulators (PGR) to induce callus formation. MS0 (0 mg L^{-1} 2,4-D), MS1 (1 mg L^{-1} 2,4-D) and MS2 (2 mg L^{-1} 2,4-D) were media used in this study. Cultured explants were subcultured biweekly.

Seed sterilization and germination were performed according to the method of Battal et al., (2019). Cotyledon (C-14), cotyledon stem (CS-14), leaf (L-14) and hypocotyls (HP-14) on the 14th day after germination of the Devegülü hollyhock and cotyledon (C-28), hypocotyl (HP-28), shoot tips (ST-28) and nodes (N-28) on the 28th day after germination of the Devegülü hollyhock were used as explant sources.

Regenerated plantlets were transferred into half strength of MS medium in glass jars to induce root formation. 1 mg L^{-1} indole butyric acid (IBA) was also added into medium to promote rooting. After explant cultivation, it was allowed to incubate in a controlled climate room with a moisture content of 50-60% at 16 ± 8 light / dark photoperiod at 24 ± 2 ºC. Cultured explants were subcultured biweekly. Each study was conducted with at least three replicates.

2.3. Micropropagation of Devegülü Plant

Nodes and shoot tips from 28-days old plants were incubated in MS medium for six weeks. Regenerated plantlets were transferred to PGR-free rooting medium (2.2 g L^{-1} MS +20 g L^{-1} sugar +4 g L^{-1} agar) after six weeks. Roots were incubated in sterile glass jars at 24 ± 2ºC in 16/8 light/dark photoperiod in the climate room. In order to promote rooting, non-rooted plants were taken into the half strength MS medium containing 1mg L^{-1} IBA.

The regenerated plantlets were removed from the tissue culture medium and cleaned from the tissue culture residues under the tap water flowing without damaging the roots. After that, they were planted in soil on the vials, covered with stretch film and incubated for three days in the controlled climate room at 24 ± 2ºC in 16/8 light/dark photoperiod. At the end of the three days, the perforations were made on the stretch film and incubated in the same

environment for one more day. After stretch film removed, Devegülü plants were irrigated with half-strength of Hoagland solution every two days regularly. Plants developed in vials were transferred to plastic pots.

2.4. Statistical Analysis

Experiments were performed with at least three replicates and statistical analyses were performed using Minitab12 One-Way ANNOVA. In the statistical comparisons between the groups, $p<0.05$ values were evaluated as statistically significant changes

3. RESULT AND DISCUSSION

3.1. Callus Formation of Devegülü Hollyhock from Different Explant Sources

As shown in Table 1 and Figure 1, the explants taken from the 14-day cotyledons produced a significantly increased callus in MS1 (91.67 ± 9.62%) and MS2 (88.89 ± 8.61%) media compared to MS0 medium. Necrosis was observed for cotyledon explants in MS0 (Figure 1. A). The rate of callus formed in 14-day cotyledon stem in MS0, MS1 and MS2 mediums did not show a statistically significant difference. CS-14 showed the highest amount of callus in MS1 (83.33 ± 16.67%). The 14-day old leaf explants produced significantly more callus in MS2 (77.7 ±25.46%) than MS0 (27.78 ± 25.46%). The 14-day hypocotyl explants produced a significantly increased callus in MS1 (88.89 ± 9.62%) and MS2 (100±0.00%) compared to MS0 (50.00 ± 25.46%).

The 28-day-old cotyledons produced a significantly increased callus in MS2 (86.67 ±23.09%) medium compared to MS0 medium (Table 2). It was not determined a statistically significant difference on callus formation in 28-day hypocotyl and shoot tip explants for different concentrations of 2,4-D hormone. Callus formation rate node explants was statistically significant in MS1 medium (94.44 ± 9.62%) compared to MS0 and MS2 media.

Explant type, age and anatomical structure play an important role in the callus formation of Devegülü plant. The variety of callus formation of different explant types and age has been reported in many other plants (Ishii et al., 2004; Dhar and Joshi, 2005; Zouzou et al., 2008). Auxins and cytokinins are plant growth regulators were commonly used together or separately in plant tissue culture (Gang et al., 2003). Auxins have a significant effect on callus induction (Hosseini et al., 2017). Different plant species or genotypes react differently to different PGRs (Pakseresht et al., 2016). Although 2,4-D is a widely used auxin in plant tissue culture, high doses cause an increase in cell division and cell elongation (Pakseresht et al., 2016). Many factors, such as genotype, explant type, tissue culture medium, plant growth regulators and

elicitors, can affect tissue culture responses, embryonic callus formation and plant regeneration (Chenar et al., 2015; Pakseresht et al., 2016). 2,4-D hormone has been widely used to induce callus formation from the most of the plant families and also Malvacea family (Zhang, 2000; Wang et al., 2004; Sun et al., 2005; Zouzou et al., 2008; Darvishi et al., 2014; Chenar, 2015). In this study, the use of 1 mg L^{-1} and 2 mg L^{-1} 2,4-D was consistent with previous studies. Different concentrations of 2,4-D (1 mg L^{-1} and 2 mg L^{-1}) were used to form callus from different explant sources of the Devegülü plant. While Hosseini et al., (2017) got the best result as 82.98% in shoot tip explants within 2,4-D and KIN-containing media during the studies of callus formation of shoot, root and leaf explants from *Althaea digitata* from Malvacea family. Similarly, we found callus formation rate as 85.83 % for shoot tip explants in MS0 and MS1 media. Compatible with our results, Rauol et al., (2010) obtained the best results in hypocotyl and cotyledon explants incubated in medium containing 2.4-D and TDZ in their callus formation studies with *Hibiscus sabdariffa* belonging to Malvacea family. Moreover, Pakseresht et al. (2016), reported that there was no significant difference in terms of callus induction and callus growth rate between hypocotyl and leaf explants, and that the amount and type of PGR and the explant type had a significant effect on callus induction and callus growth rate. In accordance with our study, Munir et al. (2012) obtained the best result in 2,4-D applied cotyledon explants to form callus to cotyledon explants of *Alcea rosea* in their PGR applied studies with different concentrations and combinations. This study is consistent with the studies indicating that cotyledon has the potential to generate more callus (Zhang 2000; Zouine and El-Hadrami, 2004; Dhar and Joshi, 2005; Michel et al., 2008; Raoul et al. 2010).

Table 1. Callus formation rate (%) of 14 days old explants

	MS0 ± SD	**MS1± SD**	**MS2± SD**
C-14	0.00 ± 0.00 [a]	91.67 ± 9.62 [b]	88.89 ± 8.61 [b]
CS-14	53.13 ± 23.66 [a]	83.33 ± 16.67 [a]	61.11 ± 9.62 [a]
HP-14	50.00 ± 25.46 [a]	88.89 ± 9.62 [b]	100 ± 0.00 [b]
L-14	27.78 ± 25.46 [a]	72.22 ± 9.62 [b]	77.78 ±25.46 [ab]

Abbreviations: C-14: 14-day-old cotyledon; CS-14: 14-day-old cotyledon stem; HP-14: 14 days of hypocotyl; L-14: 14 days old leaf; MS0: hormone-free MS; MS1: 1mg L^{-1}2,4-D added to MS; MS2: 2mg L^{-1} 2,4-D added MS; SD; standard deviation. Different letters on the same row means that there was a statistically significant difference between averages $p<0.05$.

Table 2. Callus formation rate (%) of 28 days old explants

	MS0 ± SD	MS1± SD	MS2± SD
C-28	0.00 ± 0.00 [a]	32.50 ±30.31[ab]	86.67 ±23.09 [b]
HP-28	55.56 ± 25.46 [a]	83.33 ± 16.67 [a]	83.33 ± 28.87 [a]
ST-28	85.83 ± 18.93 [a]	85.83 ± 18.93 [a]	73.33 ± 11.86 [a]
N-28	55.56 ± 25.46 [a]	94.44 ± 9.62 [b]	50.00 ± 0.00 [a]

Abbreviations: C-28; 28-day-old cotyledon, HP-28; 28-day-old hypocotyl, ST-28; 28-day-old shoot tip, N-28; 28-day-old node; MS0: hormone-free MS; MS1: 1mg L^{-1}2,4-D added to MS; MS2: 2mg L^{-1} 2,4-D added MS; SD; standard deviation. Different letters on the same row means that there was a statistically significant difference between averages $p<0.05$.

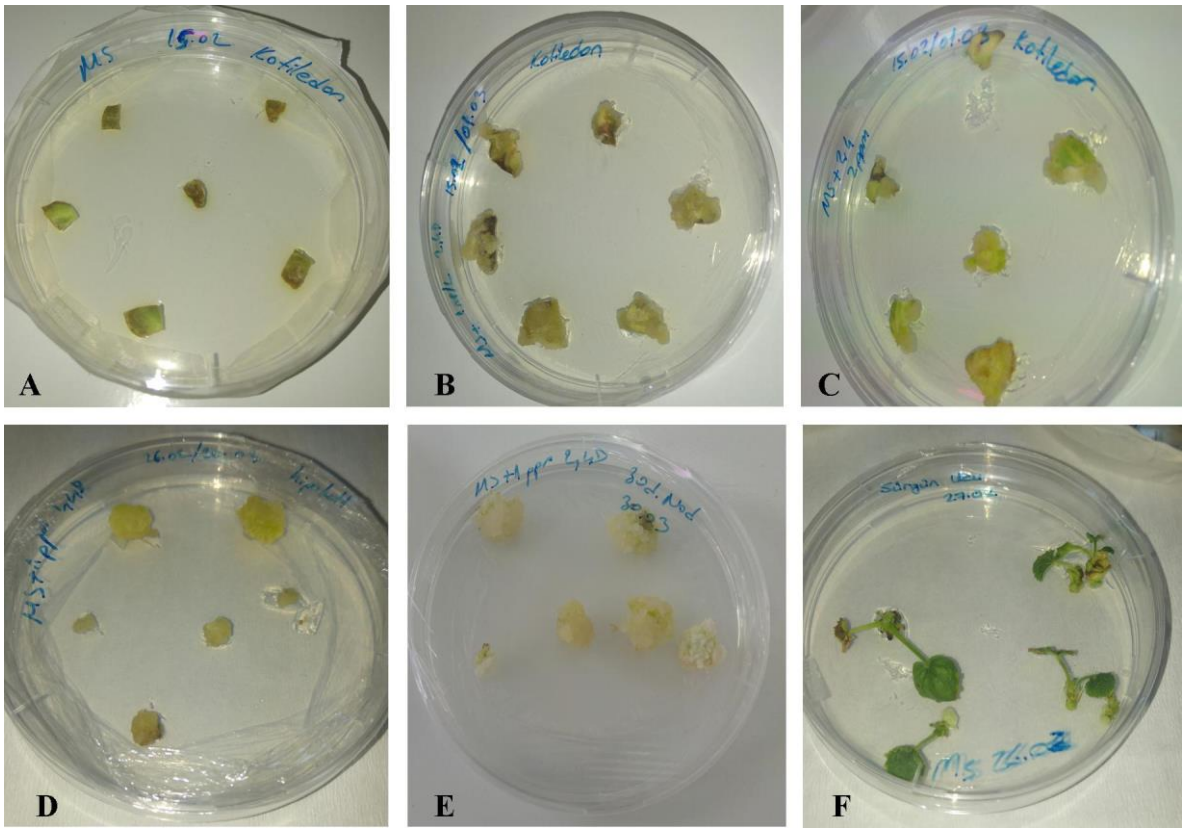

Figure 1. Callus formation from different explants of Devegülü hollyhock. Cotyledon explants with necrosis in the MS medium (A). Callus formation from cotyledon explants in the MS1 medium (B) and MS2 medium (C). Callus formation from the hypocotyl (D) and nodes (E) in MS1 medium. Shoot tips in MS medium.

3.2. Regeneration of Devegülü plant

Studies with some plants belonging to the Malvacea family, depending of callus formation and regeneration response on explant age and type, plant genotype, medium composition and other physical culture conditions were reported (Hemphill et al., 1998; Luo

et al., 2000; Özyiğit et al., 2007). In this study, the regenerated plantlets were obtained from shoot tip and nodes incubated in MS0 medium for 6 weeks (Figure 2 and 3). All of the nodes and shoot tip explants were directly regenerated (Figure 2). At the end of 6 weeks, regenerated plantlets were taken into the root formation medium (Figure 3). In previous studies with different plants such as rose (Sauer et al., 1985), blueberries (Tetsumura et al., 2008), mint (Fadel et al., 2010), half-strength of MS environment was reported to give successful results for rooting. Regarding the regeneration of Devegülü plant, it was determined that half-strength MS medium was suitable for rooting compatible with literature. 44.4 % of micro-propagated node explants produced two or more shoots in hormone free MS0 medium (Figure 2). The number of shoots per explant was 1.61. Micropropagation from node was accomplished for Devegülü plant. Although micropropagated node plants were transferred to vials, 5.6% of them could be transferred to plastic pots. Kozai (1995) described the acclimatization as the climatic adaptation of the plant before it was put into a new environment. During the stage of acclimatisation of micropropagated plants taken from tissue culture, ambient humidity and temperature should be optimized and brought to a position where the plant can survive. High relative humidity in vitro conditions adversely affect plant quality. In tissue culture, a few plants grown in the environment of relative humidity was able to survive under *ex vitro* conditions (Özkaynak and Samancı, 2005). Micropropagation was achieved at shoot tips at 12.5 % rate producing more than one shoot per plantlet in hormone free MS0 medium. The number of shoots per explant was determined as 1.125. 20% of plantlets derived from shoot tip explants were transferred into soil (Figure 4). The 28-day shoot tip and nodes are thought to be successful in regeneration due to meristematic cell density. While Özyiğit (2008) regenerated cotton plant from the same family as Devegülü plant in medium containing 0.1 mg L^{-1} KIN (kinetin)+1 g L^{-1} PVP (polyvinylpyrrolidone), Sivanesan and Jeong (2007) regenerated *Sida cordifolia* Linn (Malvacea) in 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} NAA containing medium.

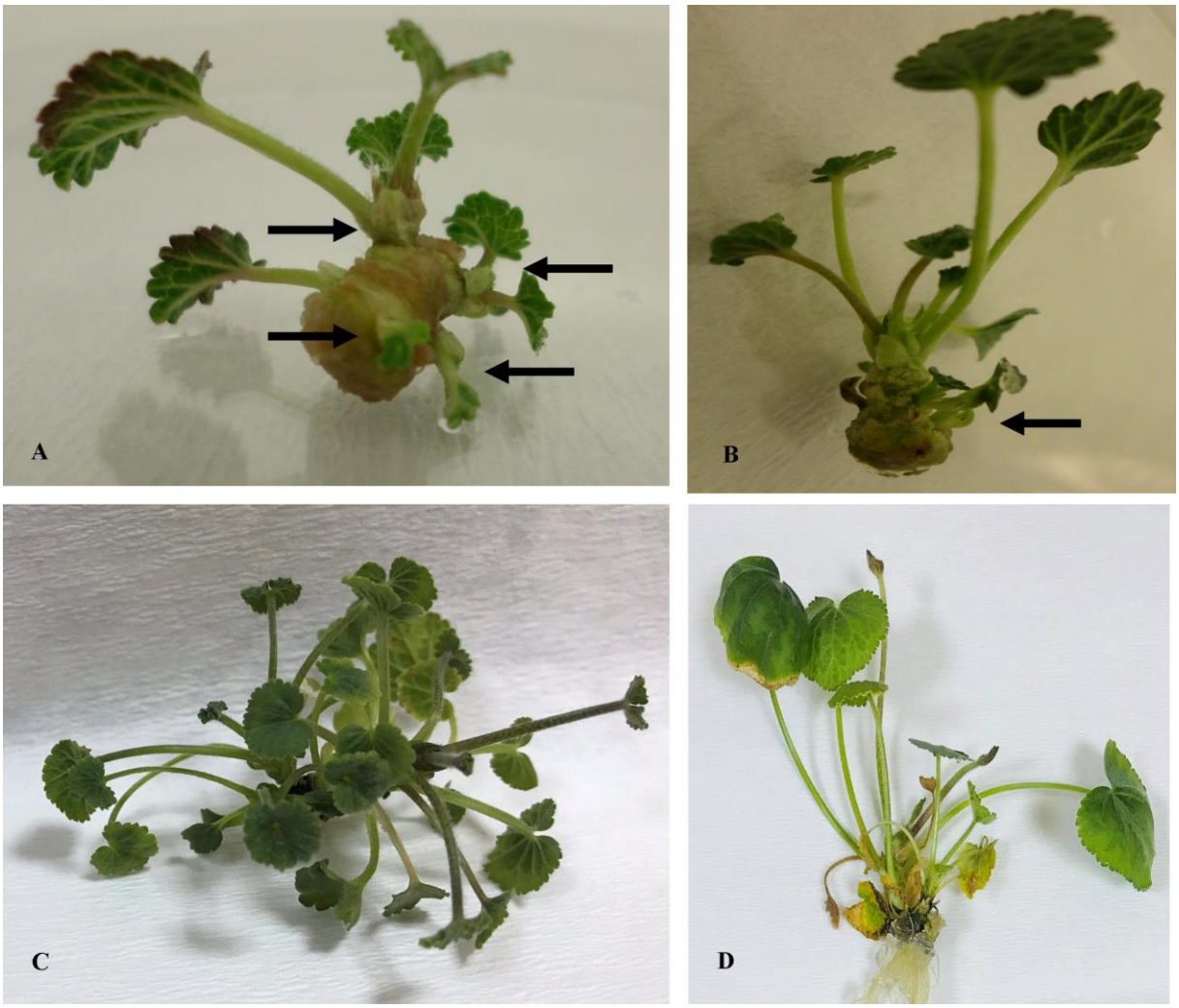

Figure 2. Micropropagation of devegülü hollyhock from node (A and C) and shoot tip (B and D). Arrows show new shoots.

Figure 3. Root formation of devegülü hollyhock. Unrooted plantlets (A), root formation after IBA treatment (B) and rooted plantlets from shoot tip explants (C) (Line is 10 cm).

Figure 4. Soil transfer and accilimization. Directly regenerated plantlets from shoot tip (A and B), regenerated plantlets from node explants (C and D).

4. CONCLUSION

With this study, in vitro regeneration of Devegülü hollyhock was achieved using as explant source of shoot tips and nodes and regenerated plants were successfully transferred into soil. Additionally, high callus formation rate and regeneration rate were observed. Moreover, micropropagation of the shoot tip and nodes was successfully achieved for Devegülü. This study could be a pioneer for the other *Alcea* species.

ACKNOWLEDGEMENTS

We would like to thank Dr. Abdullah Dalar and Dr. Muzaffer Mükemre for their help in collecting and identifying the seeds of Devegülü. This study was supported by Van Yüzüncü Yıl University Scientific Research Projects Department (FDK-2017-6160).

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