DOI: 10.17707/AgricultForest.61.4.16

Virginia SARROPOULOU, Eleni MALOUPA[1]

EFFECT OF EXOGENOUS DIKEGULAC ON IN VITRO SHOOT PROLIFERATION OF *Sideritis raeseri* L. – GREEK MOUNTAIN TEA SPECIES

SUMMARY

In the present study, the effects of 8 dikegulac concentrations (0, 10, 25, 50, 100, 250, 500, 1500 μM) on *in vitro* shoot proliferation of the Greek mountain tea species "*Sideritis raeseri* L." were investigated. The culture medium used was the MS (Murashige and Skoog) supplemented with 30 g/l sucrose and 3 g/l Phytagel. After 5 weeks of culture in the proliferation medium, shoot number/explant was greatest (5.42) and slightly increased compared to the control (4.09 shoots/explant) with 25 μM dikegulac. However, no significant changes were monitored among treatments regarding shoot length (12.48-16.11 mm). The intermediate dikegulac concentrations (50-500 μM) substantially enhanced shoot multiplication percentage in relation to the control whereas the marginal ones (10 and 1500 μM) inhibited the explants' ability to proliferate considerably. The shoot multiplication percentage was 100% when 25, 100 or 500 μM dikegulac were applied. The percentage of callus formation at the base of the explants was 100% in all treatments except for the highest dikegulac concentration (1500 μM) in which a remarkable decrease by 33.33% was recorded. Vitrification was apparent in the majority of the explants including all treatments. In specific, this symptom was less extensive (16.67-18.18%) with low dikegulac concentrations (10 or 25 μM) whereas the percentage of explants showing hyperhydration was highest (63.64%) with 50 μM dikegulac. An efficient shoot proliferation protocol under *in vitro* conditions for the Greek mountain tea species – *Sideritis raeseri* L. was established using the plant growth regulator "dikegulac" as a promoting agent with potential applications in micropropagation.

Keywords: Callus induction, dikegulac, *Sideritis raeseri* L., shoot proliferation, vitrification.

INTRODUCTION

Sideritis raeseri L. is a perennial herb of up to 40 cm. The stem is slender, pubescent, usually simple, rarely branched. The leaves are narrow, lanceolate, the 3 lower with stem and the upper with sessile. They have a green to greenish colour and are intact or slightly serrated. The calyx has a greenish color resulting in teeth and the petals of the flower are yellow. It self-sows in Parnassus, Timfristos (Velouchi) and other mountains of Aetolia, Doris and Fthiotida (Goliaris, 1984). The aromatic

[1] Virginia Sarropoulou (corresponding author: vsarrop@gmail.com), Eleni Maloupa, Institute of Breeding and Plant Genetic Resources, Hellenic Agricultural Organization - 'Demeter', Laboratory for the Conservation and Evalutation of Native and Floricultural Species, Thermi, Thessaloniki, GREECE.

Paper presented at the 6th International Scientific Agricultural Symposium "AGROSYM 2015"
Note: The authors declare that they have no conflicts of interest. Authorship Form signed online.

characteristics and its low demands make it an interesting species to be introduced as a bedding plant, a plant for rock gardens, roof gardens, restoration of degraded landscapes and to maintain slope stability. *Sideritis* plants in the wild are sexually and asexually propagated (Goliaris and Roupakias, 1997). The Greek mountain tea (*Sideritis* spp.) cultivated as a crop for tea production is propagated by seed sown in spring (Goliaris, 1984). There were no reports found in the literature on propagation of *S. raeseri*. Micropropagation of *S. raeseri* was studied as a first step for introduction in horticultural practice. The *Sideritis* genus includes more than 150 species and subspecies and is known from antiquity and folk healing to have, heating, tonic, diuretic and antioxidative properties against colds. The decoction of the aerial parts is used for diseases of the respiratory and urinary tract (Logoglu et al., 2006). Because of its beneficial properties, its over-collection in recent years in our country resulted in population reductions in many areas. Several species of the genus are considered rare and endangered. Cultivation of *Sideritis* could be a good way for Greek farmers, both by supplying the internal market and covering the needs of the demanding markets abroad, since it is a unique product. The purpose of this study was to investigate the *in vitro* propagation of the species *S. raeseri.* This method was considered necessary for its bulk vegetative propagation as the nature of the plant is such that does not give a large number of cuttings. The biggest problem is observed during rooting and acclimatization of the plantlets produced.

Dikegulac-sodium (DS)[2,3:4,6-bis-O-(1-methylethylidine)-alpha-Lxylo-2-hexulofuranosonic acid sodium salt] under the commercial name Atrimmec or Atrinal is a growth inhibitor and its action was counteracted by gibberellic acid (GA_3) in peas (*Pisum sativum* L.) (Bocion and de Silva, 1977). Furthermore, DS was shown to stimulate *in vitro* shoot multiplication of the olive cultivars Canino, Frantoio and Moraiolo but not of the cultivars Rosciola and Plantone di Moraiolo. An optimum number of olive shoots was obtained with 66.7 μM DS (Mendoza de Gyves et al., 2008). DS is transported from the leaves to the apex in very small quantities and affects only certain cell types (Arzee et al., 1977).

Very few reports exist in the literature concerning the use of DS in in vitro propagation (Mendoza de Gyves et al., 2008). In *Zantedeschia aethiopica* (calla lilly), Ebrahim (2004) noted an increase in shoot multiplication rate by increasing the DS concentration up to a certain value, above of which shoot number was similar to the control. Under certain conditions, DS acts as anti-auxins do, inhibiting polar auxin transport and reducing apical dominance (Cline, 1997).

The objective of this research was to appraise DS as a potential growth regulator which would facilitate micropropagation by evaluating its effects on shoot proliferation in the *S. raeseri* species. In this process, DS in combination with BA was used to break apical dominance and to promote lateral branching, as was shown in some other plants (Das et al., 2006; Sansberro et al., 2006).

MATERIAL AND METHODS

The experimental material was shoot tip explants from previous *S. raeseri in vitro* cultures. For the initial establishment of the plant material *in vitro* apex meristems were cut and removed from the mother plants maintained in a peat:perline (1:1) substrate in pots under unheated-greenhouse conditions. For the

disinfection of the collected plant material, shoot tips were soaked in 70% ethanol for 1 min followed by 3% NaOCl solution for 20 min under continuous stirring. The successfully established explants were sub-cultured every 4 weeks until a sufficient amount of plant material to be concentrated. The nutrient medium used was the Murashige and Skoog (MS) (Murashige and Skoog, 1962) supplemented with all the essential macronutrients, micronutrients, vitamins and amino acids. The effects of 8 DS concentrations (0, 10, 25, 50, 100, 250, 500 and 1500 μM) were studied in order to break apical dominance and to increase production of multiple shoots under *in vitro* conditions in *S. raeseri* species. All the treatments contained 2.2 μM BA, 0.05 μM indole-3-butyric acid (IBA), 0.05 μM α-naphthaleneacetic acid (NAA), 0.15 μM gibberellic acid (GA_3) and 740 μM betaine in combination with DS. The culture medium was also supplemented with 30 g L^{-1} sucrose and 3 g L^{-1} Phytagel (Gelling agent). The pH of the medium was adjusted to 5.8 before adding the gelling agent and afterwards the medium was sterilized at 121^{o}C for 20 min. All cultures were maintained in a growth chamber. The chamber was programmed to maintain 16-h light duration (40 μmol m^{-2} s^{-1}) supplied by cool white fluorescent lamps and a constant temperature of 22±2^{o}C. Apical explants with a node and two leaves (1-2 cm in length) were transferred into Magenta vessels containing 35 mL of MS culture medium. DS and GA_3 (Sigma-Aldrich chemical company USA) were filter-sterilized and added to the culture medium after autoclaving whereas BA, IBA, NAA and betaine were incorporated into the culture medium prior to autoclaving. After 5 weeks of culture, measurements were taken regarding shoot proliferation macroscopic characteristics such as shoot number/explant, shoot length and percentages (%) of shoot multiplication (%), callus formation and vitrification.

The experiment was completely randomized and analyzed by ANOVA (Analysis of Variance) using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, USA) at $P \leq 0.05$, according to Duncan's multiple range test ± S.E. in order significant differences among the treatments to be established.

RESULTS AND DISCUSSION

DS is mainly used as a plant growth regulator in order to suppress apical dominance and to enhance development of lateral buds. The effectiveness of DS in the *Sideritis raeseri* species (Greek mountain tea) as a promoting agent of shoot proliferation under *in vitro* conditions was tested. In *Sideritis raeseri* L., DS at the lowest applied concentration of 25 μM (Fig. 1c) slightly increased the number of new produced shoots per explant (5.42) without however differing from the DS-untreated explants (4.09) (Table 1, Fig. 1a). According to Sarropoulou et al. (2014), in the Gisela 6 (*Prunus cerasus* x *Prunus canescens*) cherry rootstock, the increased shoot number/ explant produced with 80 μM DS was ascribed to the high cytokinin concentrations followed by low IAA and GA_3 concentrations resulting in breaking of apical dominance, as proposed by Puglisi (2002) in the *Clematis* species, and by Ebrahim (2004) in *Zantedeschia aethiopica* cv. Spreng. Similar explanation was proposed in olives, where 66.7

and 100.5 μM DS in the cultivars Canino and Moraiolo and 33.8-100.5 μM in Frantoio cultivar increased the number of shoots/explant (Mendoza de Gyves et al., 2008). On the other hand, a reduced number of shoots was reported for the *in vitro* culture of *Vaccinium corymbosum* L. cv 'Herbert' when DS combined with 5 mg/l 2-isopentenyladenine (2ip) (Litwińczuk and Prokop, 2010).

Table 1. Effect of DS concentration in combination with 2.2 μM BA, 0.05 μM IBA, 0.05 μM NAA, 0.15 μM GA_3 and 740 μM betaine on shoot number/explant, shoot length (mm), shoot multiplication percentage (%), callus formation percentage (%) and vitrification percentage in *Sideritis raeseri* species using shoot tip explants after 5 weeks in culture.

DS (μM)	Shoot number/ explant	Shoot length (mm)	Shoot multiplication percentage (%)	Callus formation percentage (%)	Vitrification percentage (%)
0	4.09 ± 0.61 ab	12.48 ± 1.12 a	81.82 c	100 b	**54.55 e**
10	3.64 ± 0.68 ab	16.11 ± 1.17 a	72.73 b	100 b	18.18 a
25	5.42 ± 0.47 b	13.09 ± 0.53 a	**100 e**	100 b	16.67 a
50	3.73 ± 0.54 ab	13.84 ± 0.90 a	90.91 d	100 b	63.64 f
100	4.83 ± 0.52 b	14.66 ± 1.53 a	**100 e**	100 b	25.00 b
250	4.25 ± 0.55 ab	14.58 ± 0.82 a	91.67 d	100 b	41.67 d
500	4.50 ± 0.45 ab	12.99 ± 1.03 a	**100 e**	100 b	33.33 c
1500	3.00 ±0.58 a	15.60 ± 1.60 a	58.33 a	**66.67 a**	33.33 c
P-values	0.088 ns	0.263 ns	0.000***	0.000***	0.000***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ns $P \geq 0.05$; ***$P \leq 0.001$

In *Sideritis raeseri* L., DS regardless its concentration (10-1500 μM) did not influence shoot length in a considerable degree (12.48-16.11 mm) (Table 1). In a previous study conducted by Sarropoulou et al. (2014) in the cherry rootstock CAB-6P (*Prunus cerasus* L.), DS when applied at 120 μM or 150 μM concentrations diminished shoot length considerably. In addition, Mendoza de Gyves et al. (2008) reported that DS (100.5 and 133.4 μM) had a negative effect on shoot length in the olive cultivars Canino, Frantoio and Moraiolo. According to Thetford and Berry (2000), DS exerted an inhibitory effect on height in euonymus, forsythia, Chinese privet, waxleaf privet and azalea. The decrement in these species of shoot length was ascribed to the inhibition of gibberellin biosynthesis due to DS addition by inhibiting oxidation of ent-caurene to ent-kaurenic acid, which is a prerequisite for GA biosynthesis. Opposite results were reported by Ebrahim (2004) in calla, where DS (0.85-6.67 μM) increased shoot length. Considering the Gisela 6 rootstock, DS did not exert any significant effect on shoot length (Sarropoulou et al., 2014) which agrees with the results reported for the species *Christia subcordata* Moench (Whiting, 2007).

In *Sideritis raeseri* L., although the lowest (10 μM) (Fig 1b) and highest (1500 μM) (Fig. 1h) DS concentrations adversely affected the explants' ability to multiply, the intermediate DS concentrations (50-500 μM) (Fig 1d-1g) enhanced

shoot proliferation percentage (Table 1). In particular, the shoot multiplication percentage was 100% when 25, 100 or 500 μM dikegulac were incorporated into the culture medium. According to Sarropoulou et al. (2014), in CAB-6P and Gisela 6 cherry rootstocks, the percentage of explants producing new shoots was 100% in the combined effect of 4.4 μM BA with DS (250 and 500 μM). In the olive cultivar Chondrolia Chalkidikis, DS (16.9-100.5 μM) promoted the activity of cytokinin in producing new shoots whereas DS concentrations higher than 133.4 μM had a negative effect (Antonopoulou, 2009).

Callus formation at the base of the explants was evident in all DS treatments (0-1500 μM) (Table 1). The percentage of callus induction was 100% in all treatments except for the highest dikegulac concentration (1500 μM) in which a remarkable decrease by 33.33% was recorded. Vitrification or hyperhydricity of plant tissues including both shoots and leaves was apparent in the majority of the explants including all treatments. In specific, the lower DS concentrations (10 or 25 μM) ameliorated this symptom by diminishing the vitrification percentage from 54.55% in the control untreated microshoots to 16.67-18.18%. On the other hand, 50 μM DS exhibited the highest vitrification percentage (63.64%).

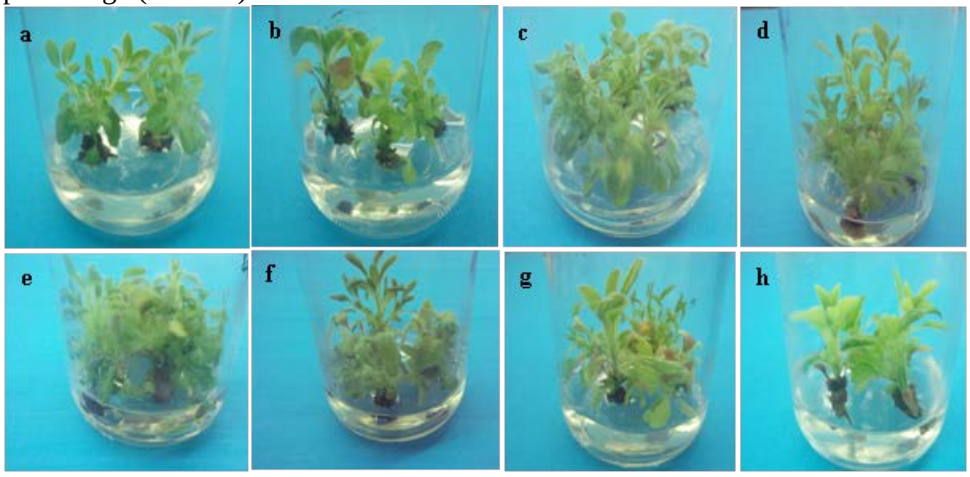

Figure 1 *In vitro* shoot proliferation of *Sideritis raeseri* shoot tip explants using DS: (a) Control (DS-free), (b) 10 μM DS, (c) 25 μM DS, (d) 50 μM DS, (e) 100 μM DS, (f) 250 μM DS, (g) 500 μM DS and (h) 1500 μM DS.

CONCLUSIONS

In the current research, DS promoted shoot propagation of *Sideritis raeseri* (Greek mountain tea from the area of Grete) under *in vitro* conditions by increasing the percentage of shoot multiplication and simultaneously by decreasing hyperhydricity of plant tissues, a common problem in micropropagation systems. To our knowledge, this is the first study which reports the use of DS as a growth regulator in *Sideritis* spp. under *in vitro* conditions with the aim to increase shoot proliferation attributes. Therefore, the use of DS

appears to be a promising substance in plant tissue culture by reducing propagation stages and time to end with rooted explants.

ACKNOWLEDGEMENTS

This research project is funded under the Action “Research & Technology Development Innovation projects (AgroETAK)”, MIS 453350, in the framework of the Operational Program “Human Resources Development”. It is co-funded by the European Social Fund and by National Resources through the National Strategic Reference Framework 2007-2013 (NSRF 2007-2013) coordinated by the Hellenic Agricultural Organisation "DEMETER" (Institute of Genetic Breeding and Natural Resources/Scientific supervisor: Dr Eleni Maloupa).

REFERENCES

Antonopoulou, C. (2009). Effect of different factors in vitrification, shoot propagation and rooting of olive (*Olea europaea* L.) *in vitro*, PhD Thesis, Aristotle University of Thessaloniki, Thessaloniki, Greece.

Arzee, T., Langenauer, H., Gressel, J (1977). Effects of dikegulac, a new growth regulator, on apical growth and development of three Compositae, Botanical Gazette, 138, 18-28.

Bocion, P.F., de Silva, W.H. (1977). Some effects of dikegulac on the physiology of whole plants and tissues: Interactions with plant hormones, Proceedings of the International Confeference on Plant Growth Substances, Lausanne, Switzerland, pp. 198-198.

Cline, M.G. (1997). Concepts and terminology of apical dominance, American Journal of Botany, 84, 1064-1069.

Das, S., Ghosh, S., Basu, P.S. (2006). Effect of dikegulac on flowering, fruit setting and development of Cucumis sativus L., Indian Journal of Plant Physiology, 11, 119-122.

Ebrahim, M.K.H. (2004). Comparison, determination and optimazing the conditions required for rhizome and shoot formation, and flowering of *in vitro* cultured calla explants, Scientia Horticulturae, 101, 305-313.

Goliaris, A. (1984). Cultivation of the mountain tea, Ministry Agric. Agritica, 16, 29-31.

Goliaris, A., Roupakias, D. (1997). Yield performance of interspecific F1 hybrids of the Greek mountain tea (*Sideritis spp*. L.), Plant Breeding, 166, 493-497.

Litwińczuk, W., Prokop, A. (2010). The usefulness of dikegulac in propagation of highbush blueberry (*Vaccinium corymbosum* L.) ‘Herbert’, Journal of Fruit and Ornamental Plant Research, 18, 85-92.

Logoglu, E., Arslan, S., Öktemer, A., Takiyan, I. (2006). Biological activites of some natural compounds from *Sideritis sipylea* Boiss, Phytotherapy Research, 20, 294-297.

Mendoza-de Gyves, E., Mira, F.R., Ruiu, F., Rugini, E. (2008). Stimulation of node and lateral shoot formation in micropropagation of olive (*Olea europea* L.) by using dikegulac, Plant Cell Tissue and Organ Culture, 92, 233-238.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiologia Plantarum, 15, 473-497.

Puglisi, S.E. (2002). Use of plant growth regulators to enhance branching of *Clematis* spp, Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Sansberro, P., Mroginski, L., Bottini, R. (2006). Stimulation of lateral branch formation on *Ilex paraguariensis* (Aquifoliaceae) seedlings, Austalian Journal of Experimental Agriculture, 46, 707-710.

Sarropoulou, V., Dimassi-Theriou, K., Therios, I. (2014). Dikegulac-sodium effect on micropropagation and biochemical parameters in the cherry rootstocks CAB-6P and Gisela 6, Turkish Journal of Agricultural and Natural Sciences, Special Issue 2, 1713-1726.

Thetford, M., Berry, J.B. (2000). Response of five woody landscape plants to primo and pruning, Journal of Environmental Horticulture, 18, 128-132.

Whiting, P.A. (2007). Commercial production of *Christia subcordata* by establishing cultural practices and by applying plant growth regulators, MSc, B.S.C.E. Florida A & M University 1995, B.S.A. University of Georgia 2006, Athens Georgia 2007.